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# Mechanisms driving tuberculosis susceptibility and vaccine efficacy in HIV/AIDS and type 2 diabetes

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The Australian Institute of Tropical Health and Medicine (AITHM) College of Public Health, Medical and Veterinary Sciences (CPHMVS) James Cook University Australia

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I dedicate this thesis to my parents for their constant support and unconditional love.

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### **Statement of Ethics and Biosafety Approvals**

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the James Cook University (JCU) Research Code based on National Health and Medical Research Council's (NHMRC) The Australian Code for the Responsible Conduct of Research (2018). The proposed research methodology received clearance from the JCU Experimentation Ethics Review Committee (Approval #A2400 and A2403) and Institutional Biosafety Committee (Approval #JCUIBC-160922-014 & #JCUIBC-170210-006).

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## **Statement of Contribution of Others**

I declare that all persons whom have contributed to the thesis have been included as coauthors for published papers or are acknowledged below:

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4	Sathkumara H, Muruganandah V, Cooper M, Alim A, Field M, Rush CM, Govan B, Henning L, Ketheesan N, Brosch R, Kupz A. (2020). Mucosal delivery of ESX-1- expressing rBCG strains confer superior immunity against TB in a mouse model of type 2 diabetes. <i>Proc. Natl. Acad. Sci. U.S.A</i> ( <i>PNAS</i> ). DOI: 10.1073/pnas.2003235117	A.K. and N.K. conceived of the study. H.D.S., V.M., M.A.A. and A.K. performed experiments; R.B. provided BCG strains and critical intellectual input; H.D.S, A.K., M.C. and M.F. performed data analysis. N.K., B.G., C.R. and L.H. assisted with troubleshooting and intellectual input. H.D.S. and A.K. wrote the initial manuscript. All co-

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Harindra D. Sathkumara

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Date

15/06/2020

### Abstract

Tuberculosis (TB) is a disease of global significance. The causative agent *Mycobacterium tuberculosis* (*Mtb*) is a bacterium known for plaguing mankind for millennia. Despite considerable global efforts, TB remains the top infectious killer worldwide to date with nearly 10 million new active cases and 1.5 million TB-related deaths annually. Further, 1.7 billion people are believed to be latently infected representing an enormous repository of potential TB reactivation. While infections caused by drug-susceptible *Mtb* strains can be treated with long-term antibiotic therapy, multi drug-resistant (MDR) and extensive drug-resistant (XDR) *Mtb* strains requiring even longer and more complicated treatments with poor clinical outcomes are on the rise globally. In addition, the lack of protection offered by the only licensed TB vaccine Bacille Calmette Guérin (BCG), and the increasing incidence of comorbid health conditions, such as human immunodeficiency virus (HIV) co-infection and diabetes mellitus (DM) among *Mtb*-infected individuals, pose a great challenge to TB control. Therefore, rationally designed new TB vaccine candidates and improved drug regimens are urgently required to meet the challenges of global TB eradication.

HIV-coinfection is the most potent risk factor for TB. Not only does it increase the susceptibility to *Mtb* infection but also enhances TB disease progression and severity, which is associated with higher mortality among coinfected individuals. Furthermore, HIV-coinfection escalates the risk of reactivation from latent TB infection (LTBI) by 10-fold. Reactivation of LTBI is mainly driven by the disruption of *Mtb*-containing granulomas, which facilitates bacterial dissemination. HIV preferentially depletes *Mtb*-reactive CD4<sup>+</sup> T cells, thus changing the formation, organisation, caseation and cellular composition of granulomas. Using a mouse model of latent lymphatic TB, which closely resembles the reactivation dynamics of LTBI in HIV<sup>+</sup> individuals, this study attempted to investigate if the vaccination with standard or improved recombinant BCG could prevent the reactivation of LTBI in mice. Our findings suggest that BCG vaccination protects mice from developing active lung TB and that the protection was largely independent of CD4<sup>+</sup> T cells. More interestingly, both traditional subcutaneous administration and mucosal delivery of the vaccine conferred functionally similar but anatomically distinct immune protection against active TB disease in mice. Intriguingly, our data also suggest that BCG-induced immune response is unlikely to be mediated by cytotoxic CD8<sup>+</sup> T cells pointing towards a potential role of B cell and/or trained innate immunity.

In addition to HIV-coinfection, the unprecedented rise in diabetes worldwide, particularly type 2 diabetes (T2D), also increases the risk of *Mtb* infection by 3-4-fold compared to a nondiabetic individual. A robust model of murine T2D was utilised to investigate the immune response in TB/T2D comorbidity. Diabetic mice displayed a distinct transcriptome signature and a microbiota facilitating increased *Mtb* infection. We show that vaccination with ESX-1expressing BCG strains has fewer adverse effects in T2D mice, and also confers near sterile anti-TB immunity against a low-dose aerosol *Mtb* infection in both diabetic and non-diabetic aged mice. Extensive cellular phenotyping revealed that superior protection induced by mucosal delivery of ESX-1-expressing BCG strains was associated with functionally and quantitatively augmented lung and airway resident antigen presenting cells (APCs). These findings highlight the significance of incorporating *Mtb* antigens into next generation TB vaccines and warrant further investigations to decipher the role of vaccine-induced innate immune response.

We also evaluated the potential use of the first-line anti-diabetic drug metformin (MET) as host-directed therapy (HDT) and as adjunctive anti-TB therapy in the context of T2D. In line with human retrospective studies, T2D mice that received a therapeutic dose of MET showed improved lung TB disease burden and reduced organ inflammation. Strikingly, although unexpected, non-diabetic mice that were treated with the same MET dose displayed significantly increased *Mtb* burden and lung immunopathology. These pre-clinical findings emphasise the importance of investigating the divergent effects on the host-immune system in diabetics vs non-diabetics prior to considering MET as an effective HDT for TB treatment.

In summary, this research study employed two robust mouse models of HIV-like LTBI reactivation and T2D to study and to test new experimental TB vaccines and host-directed therapeutical interventions. While *Mtb*-specific CD4<sup>+</sup> T cells have long been thought to be indispensable for the protective immune response against TB, our data shows otherwise, at least in the context of vaccines. In fact, this thesis accentuates the previously underappreciated role of innate immune cells. The augmented function of lung macrophages and dendritic cells after vaccination with ESX-1-expressing BCG strains is indicative of a potential role for vaccine-induced trained immunity in myeloid cells. This thesis, thus, highlights the importance of further investigating the ability of novel experimental vaccine candidates to mount not just conventional T cell responses but also non-traditional immune responses, which could be beneficial for individuals suffering from comorbid immunosuppressive conditions.

## Table of contents

Acknowledgements	i
Statement of Access	iv
Statement of Sources	v
Statement of Ethics and Biosafety Approvals	vi
Statement of Contribution of Others	vii
Abstract	xi
Table of contents	xiii
Table of figures	xvii
List of tables	xviii
Abbreviations	xix
Chapter 1: Introduction and Literature Review	1
1.1 Tuberculosis	2
1.1.1 History of tuberculosis	2
1.1.2 Epidemiology	
1.1.3 Microbiological and genomic characteristics of <i>Mtb</i>	5
1.1.4 <i>Mtb</i> infection	6
1.1.4.1 Secretion systems and <i>Mtb</i> virulence	7
1.1.5 Diagnosis of human tuberculosis	12
1.1.6 Tuberculosis treatments	13
1.1.7 Vaccines	15
1.1.8 Immunity to <i>Mtb</i>	17
1.1.8.1 Innate immunity	17
1.1.8.1.1 Innate recognition of <i>Mtb</i>	17
1.1.8.1.2 Macrophages	19
1.1.8.1.5 Neutrophils	
1.1.8.1.4 Natural Killer cells	
1 1 8 1 6 Dendritic cells	
1 1 8 1 7 Mth antigen processing and presentation	
1 1 8 2 Adaptive immunity	24
1 1 8 2 1 Cell mediated immunity to <i>Mth</i>	26
1.1.8.2.11 CD4 <sup>+</sup> T cells	26
1.1.8.2.1.1.1 Th1 cells	
1.1.8.2.1.1.2 Th2 cells	
1.1.8.2.1.1.3 Th17 and Th22 cells	
1.1.8.2.1.1.4 T <sub>reg</sub> cells	29
1.1.8.2.1.1.5 CD4 <sup>+</sup> memory T cells	30

1.	1.8.2.1.2 CD8 <sup>+</sup> T cells	. 31
1.1.	8.2.2 Humoral immunity to <i>Mtb</i>	. 32
1.1.9	Latent tuberculosis infection and the role of the granuloma	. 33
1.1.10	Immune evasion	. 36
1.1.11	Microbiota and TB	. 38
1.2 TB/	HIV comorbidity	. 40
1.2.1	Pathophysiology of HIV	. 40
1.2.2	<i>Mtb</i> /HIV co-infection	. 42
1.2.3	Animal models of <i>Mtb</i> /HIV co-infection	. 43
1.3 TB/	T2D comorbidity	. 44
1.3.1	Diabetes mellitus	. 44
1.3.1.1	Epidemiology	. 44
1.3.1.2	Risk Factors	. 45
1.3.1.3	Pathophysiology of T2D	. 45
1.3.1.4	Diabetes treatments	. 47
1.3.2	The double burden of TB/T2D	. 48
1.3.2.1	Epidemiology	. 48
1.3.2.2	Impaired anti-TB immunity in diabetes	. 49
1.3.3	Animal models of diabetes	. 52
1.4 Proj	ect aims	. 54
Chapter 2: M	aterials and Methods	. 55
2.1 Bac	teria	. 56
2.1 Bac 2.1.1	eria Bacterial strains	. 56
2.1 Bac 2.1.1 2.2 Exp	Bacterial strains erimental animals	. 56 . 56 . 57
2.1 Bac 2.1.1 2.2 Exp 2.2.1	teria Bacterial strains erimental animals Mice – husbandry and housing	. 56 . 56 . 57 . 57
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection	. 56 . 56 . 57 . 57 . 58 . 58
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3 2.2.4	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration.	. 56 . 56 . 57 . 57 . 58 . 58 . 59
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3 2.2.4 2.2.5	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration <i>In vivo</i> CD4 <sup>+</sup> T cell depletion	. 56 . 56 . 57 . 57 . 58 . 58 . 59 . 59
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3 2.2.4 2.2.5 2.3 Hist	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration <i>In vivo</i> CD4 <sup>+</sup> T cell depletion ology	. 56 . 57 . 57 . 58 . 58 . 59 . 59 . 59
<ul> <li>2.1 Bac</li> <li>2.1.1</li> <li>2.2 Exp</li> <li>2.2.1</li> <li>2.2.2</li> <li>2.2.3</li> <li>2.2.4</li> <li>2.2.5</li> <li>2.3 Hist</li> <li>2.4 Dete</li> </ul>	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration <i>In vivo</i> CD4 <sup>+</sup> T cell depletion ology ermination of organ bacterial loads	. 56 . 57 . 57 . 58 . 58 . 59 . 59 . 59 . 60
<ul> <li>2.1 Bac</li> <li>2.1.1</li> <li>2.2 Exp</li> <li>2.2.1</li> <li>2.2.2</li> <li>2.2.3</li> <li>2.2.4</li> <li>2.2.5</li> <li>2.3 Hist</li> <li>2.4 Dete</li> <li>2.5 Dete</li> </ul>	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration <i>In vivo</i> CD4 <sup>+</sup> T cell depletion ology ermination of organ bacterial loads ermination of blood cytokine levels	. 56 . 57 . 57 . 58 . 58 . 59 . 59 . 59 . 60 . 60
<ul> <li>2.1 Bac</li> <li>2.1.1</li> <li>2.2 Exp</li> <li>2.2.1</li> <li>2.2.2</li> <li>2.2.3</li> <li>2.2.4</li> <li>2.2.5</li> <li>2.3 Hist</li> <li>2.4 Dete</li> <li>2.5 Dete</li> <li>2.6 Flow</li> </ul>	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration <i>In vivo</i> CD4 <sup>+</sup> T cell depletion ology ermination of organ bacterial loads ermination of blood cytokine levels	. 56 . 57 . 57 . 58 . 59 . 59 . 59 . 60 . 60
<ul> <li>2.1 Bac</li> <li>2.1.1</li> <li>2.2 Exp</li> <li>2.2.1</li> <li>2.2.2</li> <li>2.2.3</li> <li>2.2.4</li> <li>2.2.5</li> <li>2.3 Hist</li> <li>2.4 Dete</li> <li>2.5 Dete</li> <li>2.6 Flow</li> <li>2.6.1</li> </ul>	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration <i>In vivo</i> CD4 <sup>+</sup> T cell depletion ology ermination of organ bacterial loads ermination of blood cytokine levels v cytometry Single cell preparation	56 57 57 58 59 59 60 60 60
<ul> <li>2.1 Bac</li> <li>2.1.1</li> <li>2.2 Exp</li> <li>2.2.1</li> <li>2.2.2</li> <li>2.2.3</li> <li>2.2.4</li> <li>2.2.5</li> <li>2.3 Hist</li> <li>2.4 Detc</li> <li>2.5 Detc</li> <li>2.6 Flow</li> <li>2.6.1</li> <li>2.6.1.1</li> </ul>	teria Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration <i>In vivo</i> CD4 <sup>+</sup> T cell depletion ology ermination of organ bacterial loads ermination of blood cytokine levels v cytometry Single cell preparation Lung	56 57 57 58 59 59 59 60 60 60
<ul> <li>2.1 Bac</li> <li>2.1.1</li> <li>2.2 Exp</li> <li>2.2.1</li> <li>2.2.2</li> <li>2.2.3</li> <li>2.2.4</li> <li>2.2.5</li> <li>2.3 Hist</li> <li>2.4 Dete</li> <li>2.5 Dete</li> <li>2.6 Flow</li> <li>2.6.1</li> <li>2.6.1.1</li> <li>2.6.1.2</li> <li>2.6.1</li> </ul>	teria         Bacterial strains         erimental animals         Mice – husbandry and housing         Induction and confirmation of type 2 diabetes         Immunization and infection         Metformin and isoniazid administration         In vivo CD4 <sup>+</sup> T cell depletion         cology         ermination of organ bacterial loads         ermination of blood cytokine levels         v cytometry         Single cell preparation         Lung         Intra-airway luminal cells	56 57 57 58 59 59 60 60 60 60
<ul> <li>2.1 Bac</li> <li>2.1.1</li> <li>2.2 Exp</li> <li>2.2.1</li> <li>2.2.2</li> <li>2.2.3</li> <li>2.2.4</li> <li>2.2.5</li> <li>2.3 Hist</li> <li>2.4 Detc</li> <li>2.5 Detc</li> <li>2.6 Flow</li> <li>2.6.1</li> <li>2.6.1.2</li> <li>2.6.1.3</li> <li>2.6.2</li> </ul>	teria         Bacterial strains         erimental animals         Mice – husbandry and housing         Induction and confirmation of type 2 diabetes         Immunization and infection         Metformin and isoniazid administration         In vivo CD4 <sup>+</sup> T cell depletion         ology         ermination of organ bacterial loads         ermination of blood cytokine levels         v cytometry         Single cell preparation         Lung         Intra-airway luminal cells         Lymph nodes and spleens	56 57 57 58 59 59 59 60 60 60 60 61 61
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3 2.2.4 2.2.5 2.3 Hist 2.4 Deta 2.5 Deta 2.6 Flow 2.6.1 2.6.1.1 2.6.1.2 2.6.1.3 2.6.2 2.6 3	teria         Bacterial strains         erimental animals         Mice – husbandry and housing         Induction and confirmation of type 2 diabetes         Immunization and infection         Metformin and isoniazid administration         In vivo CD4 <sup>+</sup> T cell depletion         ology         ermination of organ bacterial loads         ermination of blood cytokine levels         v cytometry         Single cell preparation         Lung         Intra-airway luminal cells         Lymph nodes and spleens         Surface staining         Intracellular staining (ICS)	56 57 57 58 59 59 60 60 60 60 61 61
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3 2.2.4 2.2.5 2.3 Hist 2.4 Dete 2.5 Dete 2.6 Flow 2.6.1 2.6.1.1 2.6.1.2 2.6.1.3 2.6.2 2.6.3 2.7 In v	Bacterial strains Bacterial strains erimental animals Mice – husbandry and housing Induction and confirmation of type 2 diabetes Immunization and infection Metformin and isoniazid administration. <i>In vivo</i> CD4 <sup>+</sup> T cell depletion ology ermination of organ bacterial loads ermination of blood cytokine levels v cytometry Single cell preparation Lung Intra-airway luminal cells Lymph nodes and spleens Surface staining Intracellular staining (ICS)	56 57 58 59 59 60 60 60 60 61 61 61 62 64
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3 2.2.4 2.2.5 2.3 Hist 2.4 Deta 2.5 Deta 2.6 Flow 2.6.1 2.6.1.1 2.6.1.2 2.6.1.3 2.6.2 2.6.3 2.7 In v. 2.8 Gen	Bacterial strains	56 57 57 58 59 59 60 60 60 61 61 61 62 64 65
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3 2.2.4 2.2.5 2.3 Hist 2.4 Dete 2.5 Dete 2.6 Flow 2.6.1 2.6.1.2 2.6.1.3 2.6.2 2.6.3 2.7 In v 2.8 Gen 2.9 LPS	Bacterial strains	56 57 58 59 59 60 60 60 61 61 61 61 62 64 65 65
2.1 Bac 2.1.1 2.2 Exp 2.2.1 2.2.2 2.2.3 2.2.4 2.2.5 2.3 Hist 2.4 Dete 2.5 Dete 2.6 Flow 2.6.1 2.6.1.1 2.6.1.2 2.6.1.3 2.6.2 2.6.3 2.7 In v 2.8 Gen 2.9 LPS 2.10 Cell	teria       Bacterial strains         erimental animals       Mice – husbandry and housing         Induction and confirmation of type 2 diabetes       Immunization and infection         Immunization and infection       Metformin and isoniazid administration         In vivo CD4 <sup>+</sup> T cell depletion       ology         cology       ermination of organ bacterial loads         ermination of blood cytokine levels       v cytometry         Single cell preparation       Lung         Intra-airway luminal cells       Lymph nodes and spleens         Surface staining       Intracellular staining (ICS)         tro lung cell stimulation and infection       erration of bone marrow derived macrophages and dendritic cells         instillation       enrichment	56 57 57 58 59 59 60 60 60 61 61 61 61 62 65 65

2.10	0.2 CD11c <sup>+</sup> dendritic cells	66
2.10	0.3 CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells	
2.11	T cell proliferation assay	
2.12	Determination of cellular nitrite production	
2.13	In vitro macrophage phagocytosis assay	67
2.14	DNA and RNA extraction	67
2.15	16S rRNA-based microbiome analysis	
2.16	NanoString assay	69
2.17	Statistical analysis	69

3.1	Abstract	72
3.2	Background	72
3.3	Results	76
3.3.1	1 Vaccination profiling	76
3.3.2	2 BCG vaccination prevents systemic spread of reactivated lymphatic <i>N</i> infection	<i>Mtb</i> 80
3.3.3	3 Vaccine-induced prevention of reactivation is independent of CD4 <sup>+</sup> T cells .	82
3.4	Discussion	85
3.5	Supplementary Figures and Tables for Chapter 3	88

4.1	Abstract
4.2	Background
4.3	Results
4.3.1	Murine T2D mimics increased human susceptibility to aerosol <i>Mtb</i> infection96
4.3.2	2 Compositional changes in the lung and gut microbiota following <i>Mtb</i> infection
4.3.3	3 Differential immune gene expression patterns following <i>Mtb</i> infection 101
4.3.4	ESX-1-containing BCG strains are safer than BCG in the context of T2D 101
4.3.5	5 ESX-1-containing BCG strains confer superior protection against aerosol <i>Mtb</i>
	challenge in both control and T2D mice104
4.3.6	5 Superiority of ESX-1-containing BCG strains is associated with increased age
4.3.7	7 ESX-1-containing BCG strains alter the immune cell composition and augment
	anti-mycobacterial function of APCs in the lung microenvironment 109
4.4	Discussion112
4.5	Supplementary Figures and Tables for Chapter 4 117

5.1	Abstract	132
5.2	Background	132
5.3	Results	133
5.3.	1 Murine model of T2D	133
5.3.	2 Divergent effects of metformin	134
5.4	Discussion	137
5.5	Supplementary Figures and Tables for Chapter 5	139
Chapter	6: General Discussion	143
6.1	BCG vaccination and reactivation of LTBI in the context of HIV co-infection.	
		144
6.2	New and improved anti-TB vaccines and adjunctive HDTs in the context of	
comor	bid diabetes	148
Chapter	7: References	155
Chapter	8: Appendices	197
8.1	Appendix 1: A systemic approach to simultaneously evaluate safety,	
immur	nogenicity, and efficacy of novel tuberculosis vaccination strategies	198
8.2	Appendix 2: Dysregulation of key cytokines may contribute to increased	
suscep	tibility of diabetic mice to Mycobacterium bovis BCG infection	212
83		
0.5	Appendix 3: A Systemic Review: The Role of Resident Memory T Cells in	

# Table of figures

Figure 1-1: Tuberculosis in various organs.    2
Figure 1-2: Estimated TB incidence rates in 2018
Figure 1-3: Estimated HIV prevalence in TB cases in 2018.
<b>Figure 1-4:</b> <i>Genetic organisation of esx loci and the espACD operon of Mtb.</i>
Figure 1-5: Phagosomal membrane rupture by ESX-1.    11
Figure 1-6: Overview of the cell mediated immune response in TB.    32
Figure 1-7: Structure of a typical granuloma
Figure 3-1: Schematic representation of infection model
Figure 3-2: Immune profiling prior to Mtb infection
Figure 3-3: BCG vaccination prevents reactivation of chronic lymphatic Mtb infection 81
<b>Figure 3-4:</b> <i>Vaccine-induced prevention of reactivation is independent of CD4+ T cells</i> 84
Figure 4-1: Murine T2D mimics increased human susceptibility to aerosol Mtb infection98
Figure 4-2: Changes in lung microbiota and immune-related gene expression following Mtb
infection
Figure 4-3: ESX-1-containing BCG strains are safer than BCG in the context of T2D 103
Figure 4-4: ESX-1-containing BCG strains confer a superior protection against aerosol Mtb
infection in both control and T2D mice
Figure 4-5: Superiority of ESX-1-containing BCG strains is associated with advanced age.
Figure 4-6: ESX-1-containing BCG strains alter the immune cell composition and augment
anti-mycobacterial function of innate APCs in the lung microenvironment111
<b>Figure 5-1:</b> <i>Diet induced model of murine T2D, Mtb infection and treatments</i> 134
Figure 5-2: Divergent effects of MET on T2D and non-diabetic mice

## List of tables

Table 1-1: First- and second-line anti-tuberculosis drugs.	. 14
Table 1-2: Current anti-tuberculosis vaccine candidates.	. 16
Table 1-3: Current anti-diabetic drugs	. 47
Table 2-1: Bacterial strains used in this study	. 56
Table 2-2: Mouse strains used in this study	. 57
Table 2-3: List of antibodies	. 62

## Abbreviations

°C	degrees Celsius	
%	Percent	
Ab	Antibody	
AIDS	Acquired immunodeficiency syndrome	
AEC	Airway epithelial cell	
Ag85	Antigen 85	
AGE	Advanced glycation end product	
AIM2	Absent in melanoma 2	
AITHM	Australian Institute of Tropical Health and Medicine	
AM	Alveolar macrophages	
AMP	Anti-microbial peptide	
AMPK	Adenosine monophosphate-activated protein kinase	
ANOVA	Analysis of variance	
AP-1	Adaptor protein-1	
APC	Antigen presenting cell	
ASL	Airway surface liquid	
AUC	Area under the curve	
BAL	Bronchial lavage	
BALF	Bronchoalveolar lavage fluid	
Batf	Basic leucine zipper transcription factor	
BCG	Bacille Calmette Guérin	
Be	B cell effector	
BMDC	Bone marrow derived dendritic cell	
BMDM	Bone marrow derived macrophage	
BMI	Body-mass index	
BSL	Biosafety level	
CCR	Chemokine receptor	
CFP10	Culture filtrate protein 10	
CFU	Colony forming unit	
cGAS	Cyclic GMP–AMP synthase	
CIITA	Class II trans-activators	
CIATEJ	Centro de Investigación y Asistencia en Tecnología y Diseño del Estado	

de Jalisc

CON	Control	
CTL	Cytolytic/ cytotoxic T lymphocyte	
CTLA-4	Cytotoxic T lymphocyte antigen-4	
CXR	Chest X-rays	
DC	Dendritic cell	
DC-SIGN	DC-specific intracellular adhesion molecule-3-grabbing non-integrin	
DDM	Dideoxymycobactin	
DEG	Differentially expressed gene	
DIMT2D	Diet induced murine model of type 2 diabetes	
dLN	Draining lymph node	
DM	Diabetes mellitus	
DNA	Deoxyribonucleic acid	
Ecc	ESX-conserved components	
EDD	Energy-dense diet	
ELISPOT	Enzyme-linked immunospot	
Env	Envelope	
ER	Endoplasmic reticulum	
ESAT-6	Early secretory antigenic target-6	
Esp	ESX-1 secretion-associated proteins	
FACS	Fluorescence-activated cell sorting or Flow cytometry	
FBS	Fetal bovine serum	
FDR	False discovery rate	
FFA	Free fatty acid	
FRET	Fluorescence resonance energy transfer	
g	G-force or Relative Centrifugation Force	
g	Gram	
GDM	Gestational diabetes mellitus	
GIP	Glucose-dependent insulinotropic peptide	
GLP-1	Glucagon-like peptide 1	
glpD	Glycerol-3-phosphate dehydrogenase	
GLUT4	Glucose transporter 4	
GM-CSF	Granulocyte-macrophage colony stimulating factor	
GSH	Glutathione	
GTT	Glucose tolerance test	

H&E	Hematoxylin and Eosin
HbA <sub>1C</sub>	Haemoglobin A1C
HBD	Human β-defensin
HDP	Host defence peptide
HDT	Host-directed therapy
HIF-1a	Hypoxia-inducible factor-1 alpha
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HNP	Human neutrophil peptide
HSC	Hematopoietic stem cells
i.d.	Intradermal
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravenous
IAPP	Islet amyloid polypeptide
iBALT	Inducible Bronchus Associated Lymphoid Tissue
ICAM	Intracellular cell adhesion molecule
ICS	Intracellular cytokine staining
IDF	International Diabetes Foundation
IFG	Impaired fasting glucose
IFN	
11 1 1	Interferon
IgA	Interferon Immunoglobulin A
IgA IGRA	Interferon Immunoglobulin A Interferon-gamma release assay
IgA IGRA IL	Interferon Immunoglobulin A Interferon-gamma release assay Interleukin
IgA IGRA IL ILC	Interferon Immunoglobulin A Interferon-gamma release assay Interleukin Innate lymphoid cell
IgA IGRA IL ILC IM	Interferon Immunoglobulin A Interferon-gamma release assay Interleukin Innate lymphoid cell Interstitial macrophages
IgA IGRA IL ILC IM INH	Interferon Immunoglobulin A Interferon-gamma release assay Interleukin Innate lymphoid cell Interstitial macrophages Isoniazid
IgA IGRA IL ILC IM INH iNKT cell	Interferon Immunoglobulin A Interferon-gamma release assay Interleukin Innate lymphoid cell Interstitial macrophages Isoniazid Invariant Natural killer T cell
I I I IgA IGRA IL ILC IM INH iNKT cell iNOS	Interferon Immunoglobulin A Interferon-gamma release assay Interleukin Innate lymphoid cell Interstitial macrophages Isoniazid Invariant Natural killer T cell Inducible nitric oxide synthase
I I I IgA IGRA IL ILC IM INH iNKT cell iNOS IR	Interferon Immunoglobulin A Interferon-gamma release assay Interleukin Innate lymphoid cell Interstitial macrophages Isoniazid Invariant Natural killer T cell Inducible nitric oxide synthase Insulin receptor
I I I I IgA IGRA IL ILC IM INH iNKT cell iNOS IR IRGM	InterferonImmunoglobulin AInterferon-gamma release assayInterleukinInnate lymphoid cellInterstitial macrophagesIsoniazidInvariant Natural killer T cellInducible nitric oxide synthaseInsulin receptorImmunity-related GTPase family M protein

JNK	JUN N-terminal kinase
kg	Kilogram
KIR	Killer immunoglobulin-like receptor
KO	Knockout
LAM	Lipoarabinomannan
LN	Lymph node
LPS	Lipopolysaccharides
LRA	Latency reversal agent
LRR	Leucine-rich repeats
LTBI	Latent tuberculosis infection
M-CSF	Macrophage-colony stimulating factor
mAb	Monoclonal antibody
MAIT cell	Mucosal-associated invariant T cell
ManLAM	Mannosylated lipoarabinomannan
MARCO	Macrophage receptors with collagenous structure
MDP	Muramyl dipeptide
MDR-TB	Multidrug-resistant tuberculosis
MET	Metformin
mg	Milligram
MHC	Major histocompatibility complex
MIC	Mycobacterium microti
min	Minute
Mincle	Macrophage inducible C-type lectin
MIP	Mycobacterium indicus pranii
ml	Millilitre
mLN	Mediastinal lymph node
mm	Millimetre
MMP9	Metalloproteinase 9
MOI	Multiplicity of infection
MPM	Mannosyl β-1-phosphomycoketides
MR1	Major histocompatibility complex class I-related protein
Mtb	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
mTORC2	Mammalian target of rapamycin complex 2

1

MyD88	Myeloid differentiation primary response protein 88
NAAT	Nucleic acid amplification test
Ndk	Nucleoside diphosphate kinase
NF-κB	Nuclear factor kappa B
NHMRC	National Health and Medical Research Council
NHP	Non-human primate
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NLRP	NOD-, LRR- and pyrin domain-containing protein
NO	Nitric oxide
NOD	Non-obese diabetic
NOD2	Nucleotide-binding oligomerization domain protein 2
NOS	Nitric oxide synthase
NOX	NADPH-oxidase
Nramp	Natural-resistance-associated macrophage protein
OADC	Oleic acid-albumin-dextrose-catalase
OD	Optical density
OTU	Operational taxonomy unit
OVA	Ovalbumin
p.i.	Post infection
p.v.	Post vaccination
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered solution
PCA	Principle component analysis
PCR	Polymerase chain reaction
PCT	Proximal convoluted tubule
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PI3K	Phosphoinositide 3-kinase
PMN	Polymorphonuclear neutrophil
PPAR	Peroxisome proliferator-activated receptor
PPD	Purified protein derivative

PRR	Pattern recognition receptors
PTP	Protein tyrosine phosphatases
RAG	Recombination-activating gene
RAGE	Receptor for advanced glycation end product
rBCG	Recombinant Bacille Calmette Guérin
RD1	Region of difference 1
RIN	Ribonucleic acid integrity number
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROCK	Rho-kinase
RORγ	Retinoic acid receptor related orphan receptor $\gamma$
ROS	Reactive oxygen species
rpm	Rounds per minute
RR-TB	Rifampicin-resistant tuberculosis
RT	Room temperature
SCID	Severe combined immunodeficiency
SD	Standard diet
Ser/Thr	Serine and Threonine
SIV	Simian immunodeficiency virus
SPF	Specific pathogen free
SSA	Sub-Saharan African
STAT	Signalling transducer and activator of transcription
STF	Soluble tuberculosis factor
STZ	Streptozotocin
T1D	Type 1 diabetes
T1SS	Type 1 secretion system
T2D	Type 2 diabetes
ТАР	Transporter associated with antigen processing
ТВ	Tuberculosis
ТСН	Thiophene-2-carboxylic acid hydrazide
T <sub>CM</sub>	T central memory cell
TCR	T cell receptor
Td	Tetanus-diphtheria
Tdap	Tetanus-diphtheria-acellular pertussis

TDM	Trehalose-6,6'-dimycolate
TDR-TB	Totally drug-resistant tuberculosis
$T_{\text{EM}}$	T effector memory cell
TG	Triglyceride
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
Treg	T regulatory cell
T <sub>RM</sub>	T resident memory cell
TST	Tuberculin skin test
TXNIP	Thioredoxin-interacting protein
UNT	Untreated
WHO	World Health Organisation
WT	Wild-type
XDR-TB	Extensively drug-resistant tuberculosis
ZN	Ziehl-Neelsen
α	Alpha
β	Beta
γ	Gamma
δ	Delta
μg	Microgram
μl	Microlitre

# Chapter 1

# **Introduction and Literature Review**

#### 1.1 Tuberculosis

#### 1.1.1 History of tuberculosis

Tuberculosis (TB) is an ancient infectious disease known for plaguing humankind for millennia. The existence of TB in ancient times is evident by typical vertebral lesions found in mummies from the Egyptian pre-dynastic era and the Peruvian pre-Columbian era<sup>1</sup>. Primarily a pulmonary disease, TB can however affect bones, and many other organ systems including the central nervous system (**Figure 1-1**)<sup>2</sup>. Typical clinical symptoms of TB were described during the Roman civilization<sup>3</sup>, although more precise signs and symptoms attributed to TB were only reported in the 19<sup>th</sup> century. The first factual evidence for human TB was reported from a 9,000-year-old Eastern Mediterranean settlement where *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB, was detected using molecular methods in a Neolithic infant and a woman who were estimated to be 12 months and 25 years in age, respectively<sup>4</sup>.



#### Figure 1-1: Tuberculosis in various organs.

Extensive lung pathology due to TB (A). Basal tuberculous meningitis and a fibrinous meningeal exudate in brain (B). Caseating lesions of miliary TB in the spleen (C). Chronic tuberculous pericarditis in the heart (D). Caseating tuberculous ulcers of the ileum (E). Caseating lesions in the kidney due to TB (F). Florid caseating tuberculous lesions in a lymph node (G). Miliary choroid tubercles in the eye (H). Chronic granulomatous lesions of the face (I & J). Figure adapted from Lawn, *et al.* <sup>5</sup>.

As an infectious disease, TB poses a significant threat to human health. It has claimed millions of lives throughout history, killing more people than any other infectious disease<sup>6</sup>. Intriguingly, in ancient Greece, TB was known as 'phthisis' and its clinical presentation was well understood. The great Greek physician, Hippocrates, described young adults with chest pain and coughing, frequently with blood in the sputum. Hippocrates thought the disease was broadly inherited, while Aristotle had an opposing view on its origin, strongly believing in its contagious nature<sup>2</sup>. Numerous archaeological findings provide evidences for widespread existence of TB in Europe after the fall of Roman Empire. It caused epidemics in Europe and North America during the 18<sup>th</sup> and 19<sup>th</sup> century when the TB case rate in London reached 1,000

per 100,000 population, accounting for more than 25% of deaths in Europe. The lethality of the disease earned it the title "Captain Among these Men of Death"<sup>5,6</sup>. However, as living standards improved in the 20<sup>th</sup> century, morbidity and mortality rates of TB steadily declined.

#### 1.1.2 Epidemiology

The global occurrence of TB is larger than previously estimated. Limited access to affordable health care services due to poverty is strongly associated with TB<sup>7</sup>. A total of 10 million new TB cases worldwide were estimated in 2018, of which two thirds were reported from eight countries: India, China, Indonesia, Philippines, Pakistan, Nigeria, Bangladesh and South Africa (**Figure 1-2**)<sup>8</sup>. Even though the incidence of TB is falling at about 2% per year<sup>8</sup>, the overall disease burden continues to rise due to the rapid growth of the world population<sup>5</sup>. TB was one of the top 10 causes of death worldwide and is among the leading causes of death from an infectious disease. Despite recent advances in diagnostics, treatments and control effects, TB today still kills an estimated 1.5 million people each year<sup>8</sup>.

Multidrug-resistant TB (MDR-TB) is defined as disease caused by *Mtb* bacilli that have developed resistance to at least two of the most potent anti-TB drugs, isoniazid and rifampicin<sup>9</sup>. An estimated half a million new combined cases of MDR-TB and rifampicin-resistant TB (RR-TB) were recorded in 2018<sup>8</sup>. In addition, strains resistant to isoniazid and rifampicin, plus any fluoroquinolone and at least one of three injectable second-line drugs, are known as extensively drug-resistant TB (XDR-TB)<sup>10</sup>. *Mtb* strains that are resistant to all first- and second-line drugs, termed totally drug-resistant (TDR), have also been reported in India<sup>11</sup> and South Africa<sup>12</sup>. Interestingly, India, China and the Russian Federation are among the top three countries and accounted for half of the MDR-TB cases<sup>8</sup>.



**Figure 1-2:** *Estimated TB incidence rates in 2018.* Figure adapted from Global Tuberculosis Report, 2019<sup>8</sup>.

Human immunodeficiency virus (HIV), the most potent risk factor for active TB<sup>13</sup>, caused more than 0.25 million deaths in TB patients co-infected with HIV in 2018<sup>8</sup>, representing the major cause of deaths in HIV patients in endemic countries<sup>14</sup>. Sub-Saharan Africa is severely affected and has the highest HIV/TB prevalence<sup>13</sup>. The estimated HIV/TB prevalence in 2018 is shown in **Figure 1-3**. The increased active TB incidence and higher risk of disease reactivation among comorbid individuals are discussed in Chapter 1.2.

Co-infection with HIV in TB patients and drug-resistant TB are great challenges to the global control of TB<sup>15</sup>. In addition, diabetes, smoking and heavy alcohol consumption are also among the major risk factors associated with TB. The large global prevalence of diabetes mellitus (DM), particularly type 2 diabetes (T2D), means its influence on the total TB burden is greater than HIV co-infection<sup>16</sup>. Interestingly, up to 70% of diabetics live in TB endemic countries<sup>17</sup>. Several studies have confirmed that individuals with diabetes have a threefold higher risk of developing TB<sup>18,19</sup> and ~16% of TB patients have co-morbid diabetes<sup>20</sup>. The association between diabetes and TB is discussed in detail in Chapter 1.3.



**Figure 1-3:** *Estimated HIV prevalence in TB cases in 2018.* Figure adapted from Global Tuberculosis Report, 2019<sup>8</sup>.

#### 1.1.3 Microbiological and genomic characteristics of *Mtb*

The German physician Robert Koch changed the history of TB on the 24<sup>th</sup> of March 1882 at the Berlin Society of Physiology, when he announced that he had succeeded in identifying and culturing the aetiological agent responsible for TB. The shape of the organism was described as rod-shaped bacteria appearing as bacilli and it was therefore called 'tubercle bacillus'<sup>6,21</sup>. In 1883 it was renamed as  $Mtb^{22}$ . Tubercle bacilli were originally stained using the alcohol-methylene blue staining technique developed by Karl Weigert, which was further refined by Ziehl and Neelsen in 1885. Using this new staining technique, Robert Koch could see that rod-shaped bacilli are localized either intracellularly or extracellularly within the lesions<sup>22</sup>.

The principal aetiological agent of human TB, *Mtb*, is an intracellular, aerobic, acid-fast, nonmotile, non-encapsulated and non-spore forming bacillus capable of growing in tissues with high oxygen content, such as lungs<sup>5</sup>. The *Mtb* cell wall is impermeable to dyes due to the lack of a phospholipid outer membrane, thus it is neither Gram positive nor Gram negative<sup>23</sup>. Instead, it has a high content of lipid and mycolic acid. This robust, wax-rich cell wall contributes significantly towards *Mtb*'s long term survival within host phagocytes. Approximately 250 *Mtb* genes (~30% of total genes) are believed to be associated with fattyacid synthesis or metabolism, further reflecting the necessity to survive harsh conditions. The bacterium is, however, slow growing, taking about 15-20 hours to replicate<sup>5,24</sup>.

Virulent Mtb, undoubtedly the most important bacterial human pathogen, is a member of closely related bacterial species termed the Mycobacterium tuberculosis complex (MTBC)<sup>25</sup>. MTBC comprises pathogens of both human and veterinary significance from seven phylogenetically distinct lineages associated with different geographical regions<sup>26</sup>. Species include Mycobacterium tuberculosis, Mycobacterium canettii, Mycobacterium africanum, Mycobacterium bovis, Mycobacterium microti, Mycobacterium caprae and Mycobacterium pinnipedii<sup>4</sup>. M. bovis is still the main cause of TB in animal species, and was responsible for 6% of all human TB deaths before milk pasteurisation was introduced<sup>27</sup>. The current TB vaccine strain, Bacille Calmette Guérin (BCG) derived from M. bovis, lacks an entire genomic region, known as region of difference 1 (RD1), which contains genes for secreted proteins such as 6 kDa early secretory antigenic target (ESAT-6)<sup>28</sup>. In addition to the loss of RD1, a number of other genomic and transcriptomic differences between M. bovis and BCG vaccine strains have been identified. Among many differentially expressed genes, higher levels of glpD2 which encodes glycerol-3-phosphate dehydrogenase (glpD) and downregulation of genes responsible for fatty acid modifications/ degradation such as *fadD2*, *fadE35*, and the  $\beta$ -oxidation complex genes fadAB and desaturases desA1, desA3 in BCG compared to M. bovis is notable<sup>29</sup>. mceG a gene essential for the host cell entry was also highly repressed in BCG<sup>29</sup>. Although the genus *Mycobacterium* is estimated to have arisen roughly 150 million years ago<sup>30</sup>, more modern strains of *M. tuberculosis* in MTBC appear to share a common ancestor from 15,000-20,000 years ago<sup>31</sup>. The full genome sequencing of *Mtb* (virulent laboratory strain H37Rv) has contributed immensely towards the overall understanding of Mtb's biology<sup>32</sup>.

#### 1.1.4 *Mtb* infection

Transmission of the disease was originally considered to be either inherited or congenital<sup>2</sup>, as transmission from a diseased person to a healthy person was beyond people's imagination in the ancient world. In 1865, Jean-Antoine Villemin, a French military physician, demonstrated the transmissibility of TB when the disease was successfully transmitted to laboratory rats by inoculating them with tuberculous tissue from a person who had died of TB. This landmark study clearly established the infectious nature of the disease<sup>21</sup>.

We now understand that *Mtb* is transmitted via aerosols. Inhalation and alveolar deposition of droplets containing 1-3 bacilli expelled by active TB patients establishes pulmonary  $TB^{5,33}$ . The lung acts as the main port of entry for bacilli and an important site of disease manifestation. However, extra-pulmonary TB cases are relatively low<sup>24</sup>, but can be up to >60% in some

populations including HIV-infected and other immunocompromised individuals<sup>34</sup>. Alveolar macrophages readily take up the bacteria and induce a localised pro-inflammatory response. Among immunocompetent individuals, 90% are capable of either eliminating *Mtb* or containing it without developing any clinical signs of the disease<sup>5</sup>. A cellular mass called 'granuloma' has long been considered necessary for containment of infection (a state of latent infection) with no obvious clinical signs<sup>35</sup>, where bacteria may remain dormant for decades<sup>2</sup>. Nevertheless, in a small number of latently infected people, the dormant bacteria can reactivate, replicate and disseminate, causing active disease<sup>36</sup>. Curiously, only 5–10% of otherwise healthy individuals (mainly infants and children) infected with *Mtb* develop primary active disease, possibly due to a not yet fully developed immune system<sup>37</sup>. In addition to the historically defined active and latent disease states, two extra clinical states have recently been proposed<sup>38</sup>. The addition of incipient and subclinical states to TB disease spectrum provides opportunities to further understand the disease dynamics which enables the development of new therapeutic and diagnostic interventions.

The pathogenicity of Mtb is associated with its ability to secrete mycobacterial peptides that interact with host cells<sup>2</sup>. Moreover, mycobacterial peptide-mediated host-pathogen interactions may also induce both innate and adaptive human immune responses to deploy effector mechanisms to control the infection.

#### 1.1.4.1 Secretion systems and *Mtb* virulence

Virulence factors are molecules secreted by pathogens that contribute to the enhanced pathogenicity of the organism by assisting bacteria to invade the host, cause disease and evade host defences<sup>2</sup>. Unlike in other classical bacteria, mycobacteria-associated virulence genes encode cell wall components, enzymes of lipid pathways, surface proteins, and secretion systems. Among which secretion systems are the most important category in *Mtb* virulence<sup>39</sup>.

Secretion systems are used by pathogenic bacteria to secrete proteins across phospholipid membranes into the host environment. These proteins often have a role in promoting bacterial virulence through different mechanisms. The proteins are involved in many aspects of virulence, from enhancing attachment to eukaryotic cells, to scavenging resources in an environmental niche, to directly intoxicating target cells and disrupting their functions<sup>40</sup>. Until recently only six secretion systems had been described in bacteria: type 1 secretion system (T1SS), T2SS, T3SS, T4SS, T5SS and T6SS. Evidence suggests that virulent mycobacteria have developed a unique protein secretion system specialised in transporting selected
substrates across the complex, thick mycobacterial cell envelope<sup>41,42</sup>. This system was originally designated as the Snm pathway. However, Abdallah, *et al.* <sup>43</sup> later proposed that it should be called type VII secretion system (T7SS). Although this specialised secretion system was originally identified in *Mtb*, these systems are also found in various other genera in the phylum Actinobacteria, high G+C Gram-positive bacteria such as *Streptomyces, Corynebacterium, Nocardia or Gordonia*<sup>44</sup>. Although at least 4 types of secretion systems are encoded by the *Mtb* genome, only the T7SS has been shown to be involved in virulence<sup>39</sup>.

T7SS are also called ESAT-6 protein family secretion or ESX systems in Mtb. Strikingly, up to five of these ESX transport systems are encoded by the mycobacterial genome<sup>43</sup>, among which at least three, including ESX-1, -3 and -5, are involved in full virulence<sup>45</sup>. A core set of proteins was found to be conserved in most of the five ESX systems. These proteins were termed ESX-conserved components (Ecc)<sup>39</sup>, and include tandem pairs of WXG proteins, an ATPase with an Ftsk-SpoIIIE motif and several other proteins with predicted transmembrane domains. Genes encoding for ESAT-6 and culture filtrate protein 10 (CFP10) (also known as EsxA and EsxB, respectively) are flanked upstream by pe35 and ppe68, which encode locusspecific PE and PPE proteins. Genes that encode components of Ftsk-SpoIIIE-like ATPas (i.e., EccCa and EccCb) and EccB (i.e., a membrane protein with two transmembrane domains and an AAA+ ATPase (EccA)) are found upstream of *pe35* and *ppe68*. Transmembrane proteins, EccD and EccE with 11 and 2 transmembrane domains, respectively, are encoded by genes found downstream of esxA and esxB (Figure 1-4). In addition, the ESX-1 locus encodes a subtilisin-like protease; MycP and several ESX-1 secretion-associated proteins (Esp), some of which are specific to ESX-1, whereas others have homologues in other ESX systems (i.e.,  $EspG)^{45}$ .



#### Figure 1-4: Genetic organisation of esx loci and the espACD operon of Mtb.

Spontaneous deletions in esx-1, which encodes for RD1 in the vaccine strains *M. bovis* BCG (red shading) and *Mycobacterium microti* (MIC; blue shading), are thought to be associated with attenuated virulence. RD1 contains genes for both ESAT-6 and CFP10. In addition, genes for both ESX-conserved components

(ecc) and ESX secretion-associated proteins (esp) are also located within the RD1 region. Figure adapted from Groschel, *et al.* <sup>45</sup>.

ESX-1 encodes RD1, a 9.5kb region that contains genes for ESAT-6 and CFP10, two of the dominant T cell antigenic targets and virulence proteins<sup>46,47</sup>. The absence of RD1 region in the vaccine strain has been found to be crucial for the attenuated virulence of BCG<sup>48,49</sup>. Many components of RD1 including Rv3870, Rv3871 and Rv3877 were found to be essential for the secretion of ESAT-6 and the function of the ESX-1 system<sup>41</sup>. One of the key functions of ESX-1 is its involvement in the induction of phagosomal rupture, which releases bacterial products into the cytosolic compartment of host phagocytes<sup>50</sup>. The role of ESX-3 and -5 in *Mtb* virulence has also been described to some extent. Siegrist, *et al.* <sup>51</sup> have shown that mycobacteria lacking ESX-3 were defective in acquiring iron and thus had impaired growth during macrophage infection. ESX-5 is involved in the secretion of various ESAT-6-like proteins containing Pro-Pro-Glu, Pro-Glu, and polymorphic GC-rich sequences<sup>52</sup>. Elsewhere, the same authors have shown that ESX-5 plays a role in inducing a caspase-independent cell death<sup>53</sup>.

Unlike the other well-studied secretion systems (i.e., T1SS-T6SS), data on functions and structures of ESX systems are now only starting to be generated. However, based on protein interaction data, a hypothetical model of function and structure has been proposed<sup>45</sup>. Although certain biological aspects of ESX systems are yet to be determined, the proteins encoded by ESX loci (Figure 1-4) are thought to consist of various components of the translocating complex, secreted substrates and accessory proteins, such as chaperones. The best-known ESX-1 substrates, EsxA and EsxB, depend on each other for stability and are expressed as a 1:1 EsxA-EsxB heterodimer<sup>54</sup>. Structural analysis based on X-ray crystallography data has further confirmed the existence of an EsxA-EsxB heterodimer and revealed that the binding between the two proteins is mediated by hydrophobic interactions<sup>55</sup>. While the last few amino acids of the C-terminus of EsxA are essential for virulence<sup>56</sup>, the last seven amino acids of EsxB's C-terminus were found to be essential for EsxB secretion<sup>57</sup>. A recent study by Houben, et al. <sup>58</sup> speculated that core components of ESX-1 system, such as EccB, EccD and EccE, are involved in membrane bound core structure of these ESX systems and transport substrates across the inner membrane including EccC, a putative translocase (Figure 1-5). Deletion of MycP encoded by ESX-1 locus has led to loss of ESX-1 secretion function and shown to have a second role in substrate processing (such as EspB)<sup>59</sup>. EspA, C and D are not encoded by the ESX-1 locus, but by an operon 260kb upstream of the ESX-1 locus called espACD operon

## (**Figure 1-4**)<sup>45</sup>.

As previously mentioned, ESX-1 is the most widely studied ESX system and directly involved in pathogenicity. ESX-1 lacking Mtb strains have many defects including attenuated pathogenicity and decreased intracellular replication<sup>46</sup>. Mtb is known to interfere with some immune cell effector mechanisms such as phagosome maturation and phagolysosome formation (see Chapter 1.1.10 for more details). Phagosomal rupture caused by a bacterium was first reported by Stamm, et al. 60 in Mycobacterium marinum, a natural pathogen of fish and amphibians. Using a single-cell fluorescence resonance energy transfer (FRET)-based method, Simeone, et al. 50 recently documented the events associated with phagolysosomal rupture followed by cytosolic translocation of *Mtb* with an intact ESX-1 system. In line with previously described characteristics, strains lacking ESX-1 or RD1 were shown to remain enclosed in an intact phagosome, implying the necessity of ESX-1 for the cytosolic translocation of the bacteria<sup>50</sup>. Many studies have shown that phagosomal rupture is mediated by ESX-1 secreted ESAT-6 (EsxA), but not CFP10<sup>61,62</sup> (Figure 1-5). This system allows mycobacterial antigens such as DNA to escape into the cytosol, followed by subsequent recognition by the host immune system. Mycobacterial DNA are thought to be sensed by cytosolic DNA sensor nucleotidyltransferase cyclic GMP-AMP synthase (cGAS)<sup>45</sup>. Activation of downstream intracellular signalling pathways leads to the production of type 1 interferons (IFNs), such as IFN-B. Elevated levels of type I IFNs were seen in patients with active TB disease<sup>63</sup>. Similar to cGAS, mycobacterial DNA are also sensed by absent in melanoma 2 (AIM2), which triggers the secretion of protective cytokines, such as interleukin-1 $\beta$  (IL-1 $\beta$ )<sup>64</sup>. Intriguingly, more recent data provide evidence that *Mtb*-derived RNA could also trigger the IFN-β production through RIG-I/MAVS/IRF7 sensing pathway <sup>65</sup>. Kupz, et al. <sup>56</sup> recently reported that ESX-1 mediated cytosolic contact is essential for NOD-, LRR- and pyrin domaincontaining 3 (NLRP3)-inflammasome-mediated secretion of IL-18 by infected DCs, which leads to the rapid, non-cognate production of interferon-gamma (IFN-y) by Mtb antigenindependent memory CD8<sup>+</sup> T cells and natural killer (NK) cells. BCG strains that lack ESX-1 failed to activate the inflammasome<sup>66</sup>, further strengthening the role of ESX-1-mediated cytosolic contact in inflammasome activation.



## Figure 1-5: Phagosomal membrane rupture by ESX-1.

RD1 encoded ESAT-6 (EsxA), which is secreted as a heterodimeric complex with CFP10 (EsxB), plays a fundamental role in phagosomal membrane rupture allowing mycobacterial components to escape from hostile acidic environment to host cell cytosol. Subsequent detection of *Mtb*-associated PAMPs by intracellular PRRs triggers the downstream production of pro-inflammatory cytokines such as IFN- $\gamma$ , IL-1 $\beta$  and IL-18. Figure adapted from Groschel, *et al.* <sup>45</sup>.

Furthermore, ESX-1 products have also been found to induce the formation of granulomas<sup>45</sup>. While ESX-1-deficient *M. marinum* failed to recruit a significant number of macrophages to induce the formation of granuloma, wild type strains successfully formed granulomas with similar cellularity and architecture to those found in TB patients<sup>67</sup>.

Virulent *Mtb* have evolved different mechanisms to counteract host defence mechanisms. Reactive nitrogen species (RNS) and reactive oxygen species (ROS) are produced mainly by host macrophages and participate in protective immunity against *Mtb*<sup>68</sup>. However, resistance to host defence mechanisms is crucial for the survival of the pathogen (see Chapter 1.1.10 for more details). Proteins such as Acr1, Acr2<sup>69</sup>, Rv2136c, Rv2224c, PonA2<sup>70</sup> have been found to facilitate the bacteria, either directly or indirectly, to intervene with oxidative and nitrosative stresses. Direct participation of enzymes such as AhpC, SodC, KatG and TpX in the RNS and ROS detoxification mechanisms is indispensable for full virulence<sup>39</sup>. Phagosome arresting enables *Mtb* to survive and replicate within a non-acidified intracellular host compartment. Nucleoside diphosphate kinase (Ndk) and low-molecular weight protein tyrosine phosphatase (PtpA) are known to play a role in preventing fusion of lysosomes with phagosomes, and thus the formation of phagolysosomes<sup>71</sup>. Immune cells utilise apoptosis to eliminate infected cells. Interestingly, virulent mycobacteria strains have been found to synthesise anti-apoptosis factors which are important for survival and virulence (i.e., NuoG, SecA2/SodA, PknE, Rv3654c and Rv3655c)<sup>39</sup>.

## 1.1.5 Diagnosis of human tuberculosis

The first identification of *Mtb* using microscopy by Robert Koch in 1882 is considered the birth of TB diagnostics<sup>6</sup>. Today, acid fast staining of the bacteria and sputum smear microscopy remains the primary diagnostic method for TB, especially in resource poor countries<sup>72</sup>. Despite the higher specificity of acid fast staining, the compromised sensitivity<sup>73</sup>, mostly in settings with HIV-TB co-infections<sup>13</sup>, has been a major drawback. The sensitivity of sputum smear microscopy can vary from greater than 80% to about 20%<sup>73</sup>. However, sensitivity can be improved by processing sputum samples with bleach followed by centrifugation or sedimentation while retaining a similar specificity<sup>74</sup>. Similarly, fluorescence microscopy has demonstrated an enhanced sensitivity for detecting *Mtb* with a comparable specificity to conventional acid fast staining<sup>73</sup>. However, the replacement of standard microscopy with fluorescence microscopy in low- and middle-income countries would be challenging due to increased maintenance and reagent costs.

Significantly higher sensitivity and specificity have been obtained by culturing *Mtb* from clinical specimens. However, the results can only be seen after a few weeks due to *Mtb*'s long doubling time, which could take up to 4 weeks on solid medium<sup>75</sup>. Automated liquid culture systems are the gold standard for TB diagnosis. They are expensive but significantly faster than traditional culture techniques<sup>5</sup>. A molecular line probe assay detects mutations associated with MDR-TB with 97% and 98% sensitivity and specificity, respectively, in smear positive sputum samples or culture isolates<sup>76</sup>. Nucleic acid amplification tests (NAATs) rely on amplifying a specific region of the *Mtb* complex genome. Commercially available NAATs such as GeneXpert and more recently endorsed TrueNat tests utilise PCR amplification of 16s rRNA.

However, lower sensitivity among sputum smear-negative samples has been observed. Therefore, further improvements are needed, specifically in sensitivity for NAATs to be used for TB diagnosis in resource poor countries<sup>77</sup>. Despite their poor performance in TB diagnosis, chest X-rays (CXR) are still being used in resource-poor settings<sup>78</sup>. Interestingly, CXR associated with acid-fast staining have substantially increased the effectiveness of TB diagnosis<sup>79,80</sup>.

Unlike in tuberculosis skin test (TST) which relies on a delayed type hypersensitivity reaction to purified protein derivative (PPD), interferon-gamma release assays (IGRA) detect the cells that produce IFN- $\gamma$  in response to *Mtb* specific antigens (rather than the direct identification of *Mtb*) and can be used effectively to diagnose latent tuberculosis infection (LTBI; refer to Chapter 1.1.9)<sup>81</sup>. High specificity levels have been reported even among BCG vaccinated groups<sup>82</sup>. Two IGRA based kits are commercially available: T-SPOT.TB<sup>®</sup> test QuantiFERON<sup>®</sup>-TB Gold In-Tube test. Among which T-SPOT.TB<sup>®</sup> shows better overall sensitivity<sup>76</sup>.

However, the lack of development of more accurate, rapid point-of-care diagnostic tests for TB which can be readily utilised in low- and middle-income countries, is a major obstacle to the diagnosis of new TB cases in these regions and globally.

#### **1.1.6 Tuberculosis treatments**

There has been a dramatic resurgence of TB cases in recent years. Treating TB caused by drugresistant strains has been more challenging than ever before. Drug-susceptible TB can be successfully treated<sup>83</sup>. The World Health Organisation (WHO) recommends that all new TB cases, irrespective of HIV status, be treated with the first-line of oral anti-TB drugs for 6 months. Isoniazid, rifampicin, ethambutol, and pyrazinamide are given during the intensive phase (first 2 months) while isoniazid and rifampicin are administered for the remaining 4 months<sup>84</sup>. First- and second-line anti-TB drugs, recommended doses and known adverse effects based on WHO TB drug classification 2011 are summarised in **Table 1-1**. MDR-TB treatment should be continued for at least 20 months (8 months of intensive phase followed by a 12-18 month of continuation phase) and include 4 different anti-TB drugs: a fluoroquinolone, an injectable aminoglycoside, a first-line anti-TB drug and a second-line anti-TB drug. Group 5 drugs can only be used if 4 other potential anti-TB drugs cannot be found<sup>85</sup>. The optimum number of anti-TB drugs, duration and dosage for XDR-TB differ between individuals and depend on the immune status, virulence and resistance of the *Mtb* strain<sup>86</sup>. In addition to the traditional anti-TB drugs listed in **Table 1-1** below, a number of new drugs have recently been approved to treat patients with drug-resistant TB including MDR- and XDR-TB. However, these new drugs; Bedaquiline, delamanid and pretomanid should only be used in combination with other drugs<sup>87</sup>. Interestingly, the new WHO TB drug classification and guidelines (2016) predominantly focus on managing drug-resistant TB cases<sup>88</sup>.

Treating HIV co-infected TB patients with combined antiretroviral and anti-TB drugs has been associated with increased incidence of adverse effects such as liver failure due to pharmacological interactions between the two types of drugs<sup>89</sup>. Therefore, further refinements are needed for an optimum antiretroviral-anti-TB therapy for HIV co-infected individuals. WHO guidelines on the management of LTBI recommends '6-month isoniazid, or 9-month isoniazid, or 3-month regimen of weekly rifapentine plus isoniazid, or 3-4 months isoniazid plus rifampicin, or 3-4 months rifampicin alone' for the treatment of LTBI<sup>90</sup>.

Various promising human recombinant cytokines such as IFN- $\gamma$ , granulocyte-macrophage colony stimulating factor (GM-CSF), IL-2, and -10 are also being assessed under preclinical evaluation to use as adjunctive therapy for TB<sup>91</sup>. Additionally, number of promising host-directed therapeutic (HDT) candidates have been tested in both pre-clinical and clinical settings to improve disease outcome in TB. This includes non-steroidal anti-inflmmatory drugs (NSAIDs), inflammatory modulators such as corticosteroids, tyrosine kinase inhibitors, vitamins such as vitamin A & D, statins and most interestingly metformin; an anti-hyperglyceamic drug (extensively reviewed by Young *et al*<sup>92</sup>).

Drug	<b>Recommended daily dosage</b>	Known adverse effects			
Group 1: First-line oral anti-tuberculosis drugs					
Isoniazid	5 mg/kg once a day	CNS toxicity; increase of liver enzymes; gastrointestinal intolerance; hepatitis; peripheral neuropathy			
Rifampicin	10 mg/kg once a day	Discolouration of body fluids; increase of liver enzymes; fever; gastrointestinal intolerance; hepatitis; hypersensitivity; thrombocytopenia			
Ethambutol	15–25 mg/kg once a day	Optic neuritis			
Pyrazinamide	25 mg/kg (range 20-30 mg/kg) once a day	Arthralgia; gastrointestinal intolerance; hepatitis; hyperuricaemia			
Group 2: Injectable anti-tuberculosis drugs					
Streptomycin, amikacin, capreomycin, kanamycin	15–20 mg/kg	Allergy; auditory and vestibular nerve damage; nausea; neuromuscular blockade; renal failure; skin rash			
Group 3: Fluoroquinolones					

Table 1-1: First- and second-line anti-tuberculosis drugs.

Ofloxacin, levofloxacin	750 (800)–1000 mg once a day	CNS toxicity; gastrointestinal intolerance; hypersensitivity			
Moxifloxacin	400 mg once a day	Dizziness; increase of liver enzymes; gastrointestinal intolerance; hallucinations; headache; QT prolongation			
Group 4: Oral bacteriostatic seco	nd-line anti-tuberculosis drugs				
Ethionamide, protionamide	500 mg for bodyweight <50 kg once a day; 750 mg for bodyweight >50 kg once a day; 1000 mg for bodyweight >70 kg once a day	CNS toxicity; gastrointestinal intolerance; hepatitis			
	500 mg for bodyweight <50 kg once a day;				
Cycloserine	750 mg for bodyweight >50 kg once a day; 1000 mg for bodyweight >70 kg once a day	CNS toxicity; dizziness; psychosis			
Para-aminosalicylic acid	8 g for bodyweight <70 kg once a day; 8–12 g for bodyweight >70 kg once a day	Gastrointestinal intolerance; hypersensitivity			
Terizidone	600 mg for bodyweight <70 kg once a day; 900 mg for bodyweight >70 kg once a day	CNS toxicity; dizziness; psychosis			
<b>Group 5</b> : Anti-tuberculosis drugs with unclear efficacy or toxicity, or under assessment for use in multidrug-resistant disease					
Clofazimine	100–300 mg once a day	Discolouration of the skin; gastrointestinal intolerance; ichthyosis			
Amoxicillin with clavulanate acid	875/125 mg twice a day or 500/250 mg three times a day	Gastrointestinal intolerance; rash			
Clarithromycin	500 mg twice a day	Gastrointestinal intolerance			
Linezolid	600 mg once a day	Anaemia; neuropathy; thrombocytopenia			
Thioacetazone	150 mg once a day	Gastrointestinal intolerance; hepatitis; hypersensitivity; vertigo			

Table adapted from Zumla, et al.<sup>89</sup>.

# 1.1.7 Vaccines

Vaccines have played a major role in safeguarding mostly children by preventing infectious diseases<sup>93</sup>. However, increased incidence of HIV co-infection, emergence of new MDR-TB and XDR-TB strains and other common comorbidities in TB such as diabetes pose a great challenge to control of TB, especially in poor countries<sup>94</sup>. Control of an intracellular respiratory pathogen like *Mtb* whose control depends on cellular immunity is extremely challenging<sup>95</sup>.

Robert Koch's attempt to devise the first ever vaccine for TB in 1890s was unsuccessful<sup>24</sup>. BCG, the only approved TB vaccine today<sup>96,97</sup>, was developed by two French scientists, Albert Calmette and Camille Guérin, in the early 1900s by attenuating (>200 passages) virulent *M. bovis*<sup>98</sup>. The vaccine strain was named after Calmette and Guérin and has been given to more than 4 billion people throughout the past 99 years with impressive safety records<sup>99</sup>. BCG prevents severe forms of miliary and meningeal TB in infants<sup>100</sup>. Although it was hoped that BCG would induce a long-lasting immunity against *Mtb*, incomplete protection against pulmonary TB in adults has been one of its major failures<sup>101</sup>. In addition, substantially higher risk of disseminated BCG disease in HIV-infected infants has been reported<sup>102</sup>. BCG is now not recommended to administer to children with HIV infection<sup>103</sup>. Despite the recent advancements in immunology and vaccinology, elimination of TB is far from a reality and BCG is clearly not the ideal vaccine for the global control of TB. Therefore, an effective vaccine against *Mtb*, not only in children but also in adults, is urgently needed to conquer the rapid emergence of new TB cases.

Development of an effective vaccine has been extremely challenging due to the lack of reliable TB biomarkers<sup>104</sup>. Improvements in safety and efficacy are expected to be achieved by introducing novel vaccine strategies and/or developing new vaccine candidates. New vaccines currently being developed are aimed at replacing BCG with much improved recombinant BCG (rBCG)<sup>105</sup>, live attenuated *Mtb* vaccines or subunit vaccines that are safe to use in immunocompromised individuals<sup>100</sup>. rBCG can be engineered as prime vaccines to express RD1 encoded *Mtb*-specific antigens such as ESAT-6, CFP10 while subunit vaccines further boost BCG, rBCG or attenuated *Mtb*-induced responses<sup>100</sup>. Some of the most advanced current vaccine candidates in clinical trials are summarised in **Table 1-2**.

The ideal next generation vaccine candidates should prevent reactivation from LTBI and achieve complete sterile eradication<sup>106</sup>, preferably by boosting immune responses towards latency associated *Mtb*-antigens such as  $Rv2660c^{107}$ . High-level protection can be achieved by being able to stimulate not only the traditional T helper 1 (Th1) subsets, but all arms of the immune system, including Th17, cytotoxic CD8<sup>+</sup> T cells, B cells and other innate cells that play important roles during the early response to *Mtb* infection.

Туре	Candidate	Description	Clinical trial status*
rBCG for pre- exposure prime vaccination	VPM1002	rBCG-expressing listeriolysin and urease deletion	Phase III ongoing
	rBCG30	rBCG-expressing Ag85B	Phase I completed
	Aeras-422	rBCG-expressing perfringolysin and Ag85A, 85B, Rv3407	Phase I terminated
	MTBVAC	Transcription factor PhoP and FadD26 deletion	Phase IIa ongoing
Viral vector for pre- exposure boosted vaccination	Oxford MVA85A/Aeras-485	Modified vaccinia Ankara-expressing Ag85A	Phase I/IIa ongoing
	Crucell Ad35/Aeras- 402	Replication-deficient adenovirus 35- expressing Ag85A, Ag85B, TB10.4	Phase I completed
	Ad5Ag85A	Replication-deficient type 5 adenovirus expressing Ag85A	Phase I ongoing

Table 1-2: Current anti-tuberculosis vaccine candidates.

Fusion protein in adjuvant for pre- exposure booster vaccination	M72/ AS01E (Tested for post exposure booster vaccine as well)	Fusion protein of M72 adjuvanted with AS01E	Phase II completed
	Hybrid4(H4):IC31	Fusion protein of TB10.4 and Ag85B adjuvanted with IC31	Phase Ib completed
	ID93/GLA-SE	Fusion protein of ID93 comprising 4 antigens associated with virulence (Rv2608, Rv3619, and Rv3620) or latency (Rv1813)	Phase IIa ongoing
	GamTBvac	Fusion protein of Ag85A and ESAT6- CFP10	Phase IIa ongoing
	AEC/BC02	Fusion protein of Ag85B and ESAT6- CFp10 combined with BCG CpG	Phase I ongoing
Fusion protein in adjuvant for post- exposure booster vaccination	H56:IC31	Fusion protein of Ag85B, ESAT-6 and Rv2660c adjuvanted with IC31	Phase IIb ongoing
Whole bacterial vaccine for therapeutic vaccination	RUTI	Detoxified Mtb in liposomes	Phase IIa ongoing
	Mycobacterium. vaccae	Inactivated M. vaccae	Phase III completed
	<i>Mycobacterium</i> <i>indicus pranii</i> (MIP; also known as Mw)	Inactivated MIP	Phase III completed
	DAR901	Inactivated whole cell <i>Mtb</i>	Phase IIb ongoing
	BCG revaccination	Whole cell M. bovis BCG	Phase II completed

Table modified from Kaufmann, *et al.*<sup>108</sup>, \* as of December 2019, <u>https://clinicaltrials.gov/</u> and TAG Pipeline Report 2019

## 1.1.8 Immunity to Mtb

#### 1.1.8.1 Innate immunity

The respiratory mucosa is the first line of defence against *Mtb* and consist of 3 layers: airway surface liquid (ASL), airway epithelial cells (AECs), and lamina propria<sup>109</sup>. ASL contains mucus, immunoglobulin A (IgA) and other innate immune factors on the luminal surface. In addition to its role as a physical barrier, AECs also recognises pathogen-associated molecular patterns (PAMPs) on the surface of *Mtb* by various pattern recognition receptors (PRRs). The antigens are then presented to immune cells residing in the lamina propria, such as mucosal-associated invariant T (MAIT) cells<sup>110</sup>, triggering a pro-inflammatory response. Bacteria that are not trapped by the respiratory mucosa along the airway will continue their journey to the alveoli, which consists of a thin layer of epithelial cells and different innate immune cells, such as alveolar macrophages (AMs), dendritic cells (DCs) and neutrophils.

# 1.1.8.1.1 Innate recognition of *Mtb*

Macrophages are professional phagocytic cells and key members of the innate immune system. *Mtb* is recognised and engulfed by AMs which reside in the pulmonary alveolus and play a

major role as the frontline barrier against most respiratory infections. A study by Giacomini, et al. <sup>111</sup> reported that exposure to *Mtb* activates both macrophages and DCs and triggers the secretion of pro-inflammatory cytokines. While tumor necrosis factor- alpha (TNF- $\alpha$ ), IL-1, and IL-6 were secreted by macrophages, infected DCs were found to produce IFN- $\gamma$  inducing cytokines, such as IL-12 and IFN- $\alpha$ . Furthermore, both mouse models of TB and AMs from patients with active TB have been demonstrated to effectively kill *Mtb* using RNS such as nitric oxide (NO) radicals<sup>112,113</sup>. However, *Mtb*, largely, if not exclusively, uses these macrophages as its preferred niche and its pathogenesis follows a relatively well-defined sequence of events during infection<sup>24,114</sup>.

Recognition of *Mtb* by innate immune cells such as macrophages and DCs is mediated by surface and intracellular PRRs, which facilitate antigen uptake. Findings by Gatfield, *et al.* <sup>115</sup> support the role of cholesterol in *Mycobacterium* spp. Uptake where depleted levels of plasma membrane cholesterol were associated with defective uptake. Recognition of terminal mannose residues in *Mtb* by mannose receptors promotes the internalization of the bacterium<sup>116</sup>. Complement receptors and scavenger receptors may also aid the uptake of *Mtb* by phagocytosis<sup>117</sup>. Besides phagocytosis, recognition of mycobacterial agonists is crucial for an effective host response. PAMPs such as lipoproteins, phosphatidylinositol mannans, lipomannan and mycobacterial DNA, are sensed by Toll-like receptors (TLRs) and trigger downstream innate immune responses establishing the initial host-pathogen interaction<sup>118</sup>.

At least 10 human and 13 murine TLRs have been characterized<sup>119</sup>. TLR2, TLR4 and TLR9 are involved in recognition of various *Mtb* components<sup>120</sup>. In contrast to gram negative bacterial lipopolysaccharides (LPS), which activates cells through TLR4<sup>121</sup>, *Mtb* cell wall glycolipids; Lipoarabinomannan (LAM) and mannosylated lipoarabinomannan (ManLAM) bind to TLR2. Soluble tuberculosis factor (STF)<sup>122</sup> and trehalose-6,6'-dimycolate (TDM, also called cord factor)<sup>123</sup> are also believed to contain TLR2 agonist properties. Subsequently, Ishikawa, *et al.* <sup>124</sup> demonstrated that macrophage inducible C-type lectin (Mincle) is also an essential receptor for TDM. Gene knockout (KO) mouse studies have revealed that TLR2-, but not TLR4-defective, mice are more susceptible to develop disease upon high-dose *Mtb* aerosol challenge due to defective granuloma formation<sup>125</sup>, implying the importance of TLR2 in protective immunity. A 38-kDa lipoprotein PhoS1 and mycobacterial heat shock proteins 60, 65, and 70 activate TLR4 signalling<sup>120,126</sup>. The protective role of TLR4 signalling was demonstrated in a study where TLR4 mutant mice developed chronic infection due to impaired

elimination of *Mtb*<sup>31</sup>. Impaired macrophage recruitment was also seen<sup>31</sup>. Heterodimers of TLR2; TLR2/TLR1 and TLR2/TLR6 also play a role in *Mtb* recognition and in signal transduction<sup>127</sup>. Unlike the other TLRs described above, TLR9 is localised intracellularly, within endosomes and phagolysosomes<sup>128</sup>, and recognises highly immunostimulatory CpG motifs in *Mtb* DNA<sup>129</sup>.

In addition to TLRs, nucleotide-binding oligomerization domain protein 2 (NOD2) and NLRP3 play a role in identifying *Mtb*-associated molecular patterns. NOD2 recognizes the peptidoglycan subunit N-glycolyl muramyl dipeptide (MDP)<sup>130</sup> and triggers the expression of the antimicrobial peptide LL37 and immunity-related GTPase family M protein (IRGM) in alveolar macrophages<sup>131</sup>, while NLRP3 inflammasome was found to be activated by ESAT-6, an ESX-1 substrate<sup>56,66</sup>. Whether ESAT-6 activates NLRP3 inflammasome directly is not known.

## 1.1.8.1.2 Macrophages

As previously discussed, macrophages (including AMs) are the main effector cells involved in the killing of *Mtb*. Recognition of bacterial components by PRRs activates macrophages followed by the induction of phagocytosis. Early production of pro-inflammatory cytokines, mainly IFN- $\gamma$  and TNF- $\alpha$ , by other innate cells and later by activated T lymphocytes during the adaptive immune phase further enhances the activation of macrophages. Rapid, sustained secretion of pro-inflammatory cytokines; TNF- $\alpha$ , IL-1, IL-6, and IL-18, by *Mtb*-infected macrophages has been observed<sup>111</sup>. In addition to the production of pro-inflammatory cytokines<sup>132</sup>, macrophages exert activation of multiple anti-mycobacterial mechanisms, including phagolysosome fusion and respiratory burst<sup>133</sup>.

Activated macrophages effectively transfer phagocytosed *Mtb* to the destructive environment of lysosomes where the bacterium will be subjected to cellular microbicides. Phagosome biogenesis and maturation is controlled by Ca<sup>2+</sup> and several regulatory molecules around the small GTP-binding proteins Rabs and their downstream effectors, which includes lipid kinases, tethering molecules, and the membrane fusion apparatus<sup>134</sup>. The process is upregulated by IFN- $\gamma$ , acting in synergy with TNF- $\alpha$  and 1,25-(OH)<sub>2</sub> vitamin D3<sup>24</sup>. The maturation process of *Mtb*contained phagosomes includes a progressive acidification through expression of H<sup>+</sup>-ATPase as well as the hydrolytic enzyme cathepsin D<sup>135</sup>. Similar to phagosomes, macrophages utilise small vesicles called endosomes to internalise microbial derived material from the extracellular environment<sup>136</sup>. Both phagosomes and endosomes carrying *Mtb* or *Mtb*-derived materials are

destined to fuse with lysosomes, forming phagolysosomes. Within the phagolysosome, mycobacteria are deprived of essential nutrients and exposed to microbicidal effectors generated by IFN-y activated macrophages. Anti-microbial peptides (AMP), reactive oxygen and nitrogen species (products of NADPH oxidase and nitric oxide synthase 2 (NOS2), respectively) are among the well-described molecules<sup>137,138</sup>. Mice lacking NOS2 have demonstrated uncontrolled growth of mycobacteria, suggesting that NO is important in protection<sup>139</sup>. Similarly, in *Phox*-deficient mice (a catalytic subunit of NADPH oxidase), a significant increase in bacterial growth was observed after an aerosol *Mtb* infection, reflecting a role of ROS, including superoxide and hydrogen peroxide, in controlling infection<sup>140</sup>. This results in extensive degradation of Mtb and other bacterial components. The natural-resistanceassociated macrophage protein (Nramp), a member of the metal ion transporter family, becomes a part of the phagosome during the phagocytosis process. Metal ions, particularly  $Fe^{2+}$ , are important in macrophage activation and toxic radical generation against pathogens. Reduced phagosomal maturation and acidification was evident in Nramp1 mutant mice<sup>141</sup>. An in vitro study has shown that 1,25-(OH)<sub>2</sub> vitamin D3 suppresses Mtb growth in the human promyelocytic cell line HL-60 by inducing NOS<sup>142</sup>. However, a report by Helming, et al. <sup>143</sup> suggests that 1,25-(OH)<sub>2</sub> vitamin D3 is a potent suppressor of IFN-y-mediated macrophage activation. A case-control study suggests that vitamin D deficiency and vitamin D receptor polymorphisms could be risk factors for TB in some populations<sup>144</sup>. Antigenic peptides from extracellular materials internalised via endocytosis may bind to major histocompatibility complex (MHC) class II molecules or human leukocyte antigen (HLA) in humans. Peptideloaded MHC class II molecules are then transported to the surface of the plasma membrane where they play a role in activating antigen-specific T cell responses against Mtb. Mycobacterial lipids generated within endosomal and phagolysomal organelles are also incorporated into vesicles, which seem to transport antigens to bystander cells such as gammadelta ( $\gamma\delta$ ) T cells and DCs<sup>24,133</sup>. Therefore, acidification involved with phagosome maturation is crucial for restriction of *Mtb* within host cells.

In addition to the production of both oxygen and nitrogen reactive species, macrophages are known to produce AMPs, also known as host defence peptides (HDPs). Most potent AMPs have been shown to interact with the *Mtb* cell envelope and the mycomembrane, which is a rigid outer membrane<sup>145</sup>. Cathelicidin, a particular human cationic antimicrobial peptide 18 kDa (hCAP18) and its antimicrobial active fragment LL-37 play important roles in the interaction between the host immune system and mycobacteria. hCAP18 and LL-37 are

produced in epithelial cells, macrophages and neutrophils in response to *Mtb* infection<sup>146</sup> and have demonstrated direct antimicrobial activity against *Mtb*<sup>147</sup>. Expression of mouse cathelicidin (CRAMP) was also shown to be induced in macrophages infected with *Mtb* via a TLR2- and TNF-dependent manner<sup>148</sup>. An *in vitro* study has shown that 1,25-(OH)<sub>2</sub> vitamin D3 induced cathelicidin expression in THP-1 cell line inhibits the growth of *M. marinum*, reflecting the important role played by 1,25-(OH)<sub>2</sub> vitamin D3<sup>149</sup>. Microscopic studies have revealed the action of CRAMP and other AMPs on the bacterial cell wall<sup>150</sup>, suggesting that the cell wall and membranes serve as important targets for these cathelicidin-based peptides. Macrophages also exert anti-mycobacterial activity by inducing apoptosis in infected cells<sup>151</sup>. Although the exact mechanisms by which autophagy eliminates intracellular *Mtb* remains to be established, induction by IFN- $\gamma$  and its downstream effector p47 GTPase LRG-47 are believed to overcome the phagosome maturation block and eliminate *Mtb*<sup>152</sup>.

#### 1.1.8.1.3 Neutrophils

Interestingly, neutrophils (also known as polymorphonuclear neutrophils; PMNs) were found to be the most commonly infected phagocytes in humans with active TB, where bacilli appear to replicate rapidly<sup>153</sup>. Neutrophils contain antimicrobial peptides with anti-tuberculosis activity, namely cathelicidin, human neutrophil peptides (HNPs) 1 and 3 and Lipocalin 2, which are involved in killing and restricting growth of  $Mtb^{154}$ . Furthermore, higher neutrophil counts were shown to be associated with protection against early infection<sup>154</sup>. Recruitment and accumulation of neutrophils at the site of infection happens quickly. Increased expression of different cytokines, chemokines and cell adhesion molecules such as IL-8, IL-17, IL-23, intercellular cell adhesion molecule (ICAM)-1, E-selectin and P-selectin contribute to the neutrophil influx<sup>155</sup>. Once neutrophils arrive at the site of infection, they directly recognise the bacteria either using the PRRs described previously, or via opsonisation followed by internalisation of Mtb. This leads to activation of neutrophils. Activated neutrophils secrete cytokines, chemokines (including IL-1, -8, -17, TNF-a, Gro-a, LTB4) and several granule products including HNPs<sup>156,157</sup>, further enhancing neutrophil recruitment. Binding of HNPs to the plasma membrane leads to leakage of the cellular contents by disrupting the membrane integrity. Free HNPs were found to be taken up by macrophages, in turn enhancing their ability to kill  $Mtb^{158}$ . In addition to killing by non-oxidative mechanisms, some studies suggest that the oxidative burst may be important in the apoptosis of Mtb-infected neutrophils<sup>159</sup>. However, in the absence of killing, neutrophils can shuttle live bacteria to more distal sites acting as a 'Trojan horse'<sup>160</sup>. Studies have suggested that neutrophil-produced IL-17 may contribute to the dissemination of *Mtb*, and anti-IL-17 treatment significantly decreased the bacterial load in mouse spleens<sup>161</sup>.

#### 1.1.8.1.4 Natural Killer cells

NK cells are granular lymphocytes that express CD16, an F<sub>c</sub> receptor, and neural cell adhesion molecule CD56. Different surface receptors on NK cells such as NKp30, NKp44 and NKp46, recognise *Mtb*-infected monocytes<sup>162</sup>. IL-12 secreted by macrophages, DCs and neutrophils, increases NK cell function and promotes the synthesis of IFN- $\gamma$ , which inhibits the growth of  $Mtb^{163}$ . IFN- $\gamma$  is known to activate NADPH-oxidase type 1 and 2 (NOX1, 2) as well as NOS2, leading to the formation of ROS and RNS, respectively<sup>162</sup>. TNF- $\alpha$ , produced by NK cells, induces the formation of ROS in macrophages through the TNF receptor 1 (TNFR1) complex<sup>164</sup>. These reactive nitrogen and oxygen species disrupt the membrane lipids, proteins, DNA, and enzymes thus inducing host cell apoptosis. Additionally, NK cells harbour granules containing granulysin, perforin and granzymes. Perforin forms pores in the target cell, facilitating the transport of granulysin and granzymes. Granzyme B in NK cells can initiate cell apoptosis through direct activation of apoptotic cysteine proteases and caspases 3 and  $7^{165}$ . Granulysin can kill extracellular Mtb on its own while perforin is required for intracellular killing. NK cells express the death receptor ligand FasL. Engagement of death receptors in target cells, such as Mtb infected macrophages, results in Fas-FasL-mediated caspasedependent apoptosis, limiting the growth of  $Mtb^{162}$ . In addition, glutathione (GSH) produced by NK cells has also been shown to play an interesting role in Mtb infection. GSH is believed to enhance the cytolytic activity of NK cells, thus limiting the growth of the bacterium through direct anti-mycobacterial activity. NK cell activity and GSH levels were found to be severely compromised in HIV-infected TB patients which may contribute to the increased susceptibility of HIV-infected individuals to Mtb infection<sup>166</sup>.

### 1.1.8.1.5 Innate-like T & Innate Lymphoid Cells

MAIT cells are a unique population of  $\alpha\beta$  T cells that reside in mucosal tissues, including the respiratory mucosa. Unlike conventional CD4<sup>+</sup> and CD8<sup>+</sup> T cells found in the adaptive phase of the immune response, MAIT cells do not depend on clonal expansion, thus triggering an immediate immune response<sup>35</sup>. MAIT cells are an early innate source of both IFN- $\gamma$  and IL-17A and were found to reduce the growth of *Mtb* in association with macrophages, *in vivo*<sup>167</sup>. Furthermore, MAIT cells recognise *Mtb*-derived antigens in the context of MHC class I-related protein 1 (MR1) and produce granzyme A and perforin, which kill bacterially sensitized

targets<sup>168</sup>. Significantly lower levels of Natural Killer T (NKT) cells have been observed in patients with active TB disease<sup>169</sup>. Invariant NKT (iNKT cells), a group of NKT cells that express an invariant T cell receptor (TCR) - $\alpha$  chain, have been shown to recognize *Mtb*infected macrophages. Activation of iNKT cells requires CD1d and leads to the production of the pro-inflammatory cytokine IFN- $\gamma^{170}$ .  $\gamma\delta$  T cells expand during early infection and limit *Mtb* growth by secreting IFN- $\gamma^{171}$ . By employing an enzyme-linked immunospot (ELISPOT) assay, Ulrichs, *et al.* <sup>172</sup> demonstrated that the production of IFN- $\gamma$  by CD1-restricted T cells in response to natural *Mtb* lipid antigens strongly correlates with previous *Mtb* infections. However, D'Souza, *et al.* <sup>173</sup> suggested that  $\gamma\delta$  T cells may not directly contribute to the protection against *Mtb*, or do so only when bacterial loads are very high.

In addition to innate-like T or unconventional T cells, innate lymphocyte cells (ILCs) are also emerging as pathogenic mediators in the lung. Interestingly, group 3 or type 3 ILCs (ILC3) are the main IL-22 producer in the lung tissue in addition to  $\gamma\delta$  T, Th17 and Th22 cells<sup>174</sup>. Moreover, following an aerosol *Mtb* infection, a rapid accumulation of ILC3 was seen in B6 mice lungs<sup>175</sup>. Ardain *et al* have also demonstrated that reduction in lung ILC3s has impaired the early immune control of *Mtb* which coincided with diminished IL-17 and IL-22 production<sup>175</sup>. Intriguingly, adoptive transfer of ILC3s improved the disease outcome in TB/T2D comorbid mice<sup>176</sup>.

### 1.1.8.1.6 Dendritic cells

PRRs such as TLRs, complement receptors, mannose receptors and dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) readily recognise *Mtb*-derived antigens and rapidly activate DCs<sup>35,177</sup>. *Mtb* stimuli upregulate DC expression of costimulatory molecules B7-1 (CD80) and B7-2 (CD86), ICAM-1 and the signalling molecule CD40<sup>178</sup>. Activated DCs internalise *Mtb* and subsequently undergo maturation. As a consequence, activated DCs migrate to lymphoid tissues where they present *Mtb*-derived antigens to T cells in the context of MHC and costimulatory molecules<sup>179</sup>, initiating the adaptive phase of the immune response<sup>180</sup>. The migration of DCs to lymphoid tissues, particularly to draining lymph nodes (dLN), is promoted by the expression of inflammatory cytokines and chemokines. Upregulation of chemokine receptor 6 (CCR6) and CCR7 in DCs allows the direct migration to dLN in response to a gradient of CCL19 and CCL21 chemokines<sup>181</sup>. Conversely, Roberts, *et al.* <sup>177</sup> reported that *Mtb* increases the time required for DCs to migrate to dLN by reducing the integrin surface expression thus delaying priming of

the *Mtb*-specific T cell response. Reduced cell migration due to decreased CCR7 expression was observed in DCs infected with some clinical *Mtb* strains<sup>182</sup>. Activation of T cells and subsequent IFN- $\gamma$  production amplify the early inflammatory response, leading to macrophage activation and removal of bacteria. Significant impaired control of bacterial replication due to defective CD4<sup>+</sup> T cell response was seen in DC depleted mice<sup>183</sup>.

Increased production of pro-inflammatory cytokines such as IL-12, TNF- $\alpha$ , IL-1 and IL-6 was observed in *Mtb*-infected DCs which may have a role in DC maturation, and possibly migration and antigen processing and presentation<sup>184</sup>. An *in vitro* study has shown that IFN- $\gamma$ -inducing cytokines, IL-12 and IFN- $\alpha$ , were expressed almost exclusively by infected DCs<sup>111</sup>. This study further suggested that IL-12, IFN- $\alpha$  and IL-18 play crucial roles in enhancing IFN- $\gamma$  production by activated T cells. Additionally, *Mtb*-infected DCs have been shown to produce elevated levels of chemokines such as CCL3, CCL4, CXCL8, CXCL9 and chemokine receptors such as CCR7, which are important for the migration of NK and T cells<sup>185</sup>.

# 1.1.8.1.7 Mtb antigen processing and presentation

T cell activation requires *Mtb*-associated antigens being presented in the context of MHC molecules and related molecules such as MR1 and CD1 by professional antigen presenting cells (APCs), such as macrophages and DCs. DCs are essential to prime naïve T cells, thus activating T cell differentiation into effector and memory T cells. Macrophages are not involved in activating naïve T cells, but rather they play a role in activating both effector and memory T cells at the site of infection. Prolonged TLR signalling has been shown to reduce the expression of MHC class II molecules in macrophages whereas this signalling stimulates DCs to undergo maturation and have shown to upregulate MHC class II and other costimulatory molecules<sup>186</sup>. Uncontrolled inflammatory responses can cause deleterious effects on host tissues. Therefore, reduced expression of MHC class II molecules by macrophages is believed to serve as a negative-feedback regulation to limit excessive tissue damage from the activation of effector T cells<sup>186</sup>.

Development of adaptive immunity to *Mtb* in humans takes up to 6 weeks, whereas in mice, an antigen specific T cell response only requires about 12 days after aerosol *Mtb* infection<sup>118,187</sup>. These intervals to initiate an adaptive immune response are longer than in other infections with pathogens such as influenza virus<sup>188</sup>. *Mtb* is a lung pathogen, but the initiation of adaptive immune response to *Mtb* (development of *Mtb*-specific T cells) occurs only in the dLN<sup>187</sup>, and requires the transport of bacteria from the lungs to the draining lymph nodes, mainly by DCs<sup>189</sup>.

In mice, the transport of *Mtb* to lymph nodes following an aerosol *Mtb* infection was shown to take 8-10 days<sup>190</sup>, in contrast to approximately 20 hours in influenza virus infection<sup>191</sup>. Why this step takes so long is unclear. However, as previously described, reduced expression of the integrin CCR7 has been observed in *Mtb*-infected DCs. Once bacteria are transported to dLNs, DCs present antigens in the context of MHC Class II to naïve CD4<sup>+</sup> T cells, resulting in antigen-specific T cell proliferation, differentiation and effector T cell trafficking to the lungs<sup>189</sup>. In addition to the initial MHC-TCR interaction, co-stimulatory signalling and early cytokines promote the differentiation of naïve CD4<sup>+</sup> T cells. IL-12 and IL-18 production by phagocytes, mainly by DCs in response to *Mtb*<sup>111</sup>, induce the differentiation of naïve CD4<sup>+</sup> T cells into the Th1 subset, which is typically the main effector T cell lineage in *Mtb* infection<sup>192</sup>.

In addition to MHC class II, Mtb antigens have been shown to access the MHC class I antigen processing and presentation pathway for activation of CD8<sup>+</sup> cells<sup>24</sup>. Precisely how pathogens such as *Mtb* residing within phagosomes, can elicit CD8<sup>+</sup> T cell responses is unclear. Several mechanisms have been proposed for how Mtb-antigens access this MHC class I pathway. A study by Mazzaccaro, et al. 193 suggests that virulent strains of Mtb may have evolved a comparable mechanism to Listeria monocytogenes, which uses a soluble listeriolysin, a poreforming hemolysin to perforate the phagosomal membrane. Lipoproteins and glycolipids generated within endosomal and phagolysomal organelles are incorporated into vesicles that seem to shuttle the antigens to bystander cells such as  $\gamma\delta$  T cells and DCs<sup>24,133</sup>. Schaible, *et al.* <sup>194</sup> demonstrated that apoptotic vesicles released during *Mtb*-induced apoptosis carry mycobacterial antigens to uninfected APCs. Subsequently, mycobacterial antigens are taken up by bystander APCs and present them to CD8<sup>+</sup> T cells in the context of MHC class I and CD1b. A recent report by Tzelepis, et al. <sup>195</sup> further supports the role of bystander DCs in engulfing apoptotic bodies by efferocytosis. The authors suggested that Mtb antigens gain access to MHC class I pathway through a mechanism called 'cross-presentation' and demonstrated the importance of annexin1, which intrinsically enhances the overall capacity of the DCs' antigen-presenting machinery. Mice lacking MHC class I pathway components, such as transporter associated with antigen processing  $(TAP)^{196}$  and  $\beta_2$ -microglobulin<sup>197</sup>, were susceptible to *Mtb* infection, suggesting the importance of MHC class I-restricted CD8<sup>+</sup> T cells in *Mtb* infection.

CD1 molecules are structurally related to MHC class I and expressed on double positive thymocytes (CD4<sup>+</sup>CD8<sup>+</sup>), peripheral APCs including DCs, monocytes and a subset of B

cells<sup>198</sup>. These  $\beta_2$ -microglobulin-associated proteins enable the recognition of a range of lipidcontaining antigens such as lipopeptide dideoxymycobactin (DDM)<sup>199</sup>, LAM<sup>200</sup> and Mannosyl  $\beta$ -1-phosphomycoketides (MPM)<sup>201</sup>. Some of the  $\gamma\delta$  T cell clones seem to recognize CD1c present on B cells and DCs<sup>202</sup>. These unconventional T cells possess a cytolytic, Th1 effector phenotype and produce granulysin, suggesting a direct functional role in antimicrobial immune responses.

#### 1.1.8.2 Adaptive immunity

## 1.1.8.2.1 Cell mediated immunity to Mtb

### 1.1.8.2.1.1 CD4<sup>+</sup> T cells

 $CD4^+$  T cells play a central role in protective immunity against *Mtb*. Recruitment of  $CD4^+$  T cells to the lung following *Mtb* infection is well-established. The  $CD4^+$  T cell phenotype is largely determined by the stimulating conditions such as the cytokine milieu.

## 1.1.8.2.1.1.1 Th1 cells

Studies have shown that *Mtb*-specific Th1 cell depletion causes the early onset of disease in individuals with LTBI who become HIV infected<sup>203</sup>. IL-12 secretion by antigen presenting DCs drives the differentiation of naïve CD4<sup>+</sup> T cells into the Th1 subset, which are potent IFN- $\gamma$ producers. In addition to IL-12, the master transcription factor T-bet, and the signalling transducer and activator of transcription 4 (STAT4) are required for Th1 differentiation<sup>204</sup>. IFN- $\gamma$  is essential for resistance to *Mtb*. Both IFN- $\gamma^{205}$  and MHC class II<sup>206</sup> deficient mice rapidly succumbed to infection. Th1 cells also produce IL-12 and TNF-a. An important function of IFN- $\gamma$  is the activation of infected macrophages, which leads to the induction of phagosome maturation, production of reactive nitrogen intermediates, and antigen presentation. Mice deficient in Th1 cytokines, mainly IFN-y, demonstrated defects in production of both RNS<sup>207</sup> and ROS<sup>208</sup> and failed to control mycobacterial infection. Seneviratne, et al. <sup>209</sup> reported a case of a disseminated Mtb infection of a woman with reduced IFN-y levels where complete healing of disseminated lesions was seen only after addition of subcutaneous IFN-y to the patient's treatment regimen. Certain mutations in IFNGR1, IFNGR2, IL12B and IL12RB1 genes have been found to result in impaired IFN-γ-mediated immunity to a spectrum of mycobacterial infections in childhood<sup>210</sup>. Th1 cytokines<sup>211</sup> and chemokines such as RANTES (CCL5)<sup>212</sup> have been attributed a role in aiding the formation and maturation of the granuloma. In addition to the NKT cells, CD4<sup>+</sup> T cells can also be a major source of GM-CSF during infections. Although the exact mechanisms remain unclear, GM-CSF is known to induce *in vitro* infected macrophages to restrict the growth of  $Mtb^{213}$ .

Some of the Th1 CD4<sup>+</sup> T cells have been shown to induce apoptosis through perforin and granulysin, Fas-FasL or TNF- $\alpha$  lytic pathways<sup>214</sup>. The Fas-FasL interaction triggers the induction of classical caspase-dependent apoptosis<sup>215</sup>, and is a major cytolytic pathway that CD4<sup>+</sup> cytotoxic T cells use to kill infected cells<sup>216,217</sup>, including macrophages<sup>218</sup>. CD4<sup>+</sup> cytotoxic cells were once thought to be a potential artefact of long-term *in vitro* culturing, but are now considered to also exist *in vivo* and may play a protective role in infections<sup>219</sup>. *In vitro* stimulation of CD4<sup>+</sup> T cells with *Mtb* antigens upregulates the expression of granzyme A and B, granulysin, perforin, and more importantly FasL. Oddo, *et al.* <sup>214</sup> have demonstrated that FasL-induced apoptosis of *Mtb*-infected macrophages is associated with substantially reduced levels of viable intracellular bacteria. However, inhibition of perforin activity, Fas-FasL interaction, or both, did not significantly affect CD4<sup>+</sup> T cell mediated restriction of *Mtb* growth<sup>220</sup>, suggesting a possible perforin and Fas/FasL independent killing mechanism.

Interestingly, there is evidence that  $CD4^+$  T cells mediate IFN- $\gamma$ -independent control of *Mtb* infection. Cowley, et al. <sup>221</sup> have shown that CD4<sup>+</sup> T cells controlled >90% of intracellular Mtb growth *in vitro* in the complete absence of IFN- $\gamma$ , possibly via a NO-dependent mechanism. Additionally, BCG-vaccinated IFN-y-deficient mice exhibited a significant protection against *Mtb*, and *in vivo* depletion of CD4<sup>+</sup> T cells abrogated this IFN- $\gamma$ -independent immunity. Furthermore, Gallegos, et al. 222 demonstrated that adoptively transferred ESAT-6-specific Th1 CD4<sup>+</sup> T cells were effective at controlling *Mtb* infection in IFN- $\gamma$ , TNF- $\alpha$ , NOS2 and PHOX deficient mice. Optimal protection to Mtb required a Th1 population regardless of their effector phenotype. Recent advances in intravascular staining techniques has enabled researchers to distinguish two *Mtb*-specific CD4<sup>+</sup> T cell populations: lung parenchymal Th1 cells (high expression of CXCR3, PD-1 and CD69) and lung intravascular Th1 cells (high expression KLRG1 and CX3CR1). Interestingly, upon stimulation, intravascular Th1 cells produce a much higher level of IFN-y. However, the adoptive transfer of *Mtb*-specific parenchymal CD4<sup>+</sup> T cells was found to induce greater protection against *Mtb* infection compared to the intravascular Th1 population<sup>223</sup>. These findings suggest that lung tissue migratory capacity, rather than IFN- $\gamma$  production, could be an important property in *Mtb*-specific CD4<sup>+</sup> T cell mediated protection. Deleterious effects of unregulated CD4<sup>+</sup>T cells have also been shown in some experiments<sup>224</sup>.

### 1.1.8.2.1.1.2 Th2 cells

Th2 cell differentiation is largely driven by IL-4 and controlled by master transcription factors GATA3 and STAT6. A Th2 immune response is characterised by the production of IL-4; the signature cytokine of Th2 cells which is involved in activating B cells to induce antibodies. In addition, IL-5, IL-10 and IL-13 are also produced by Th2 cells<sup>225</sup>.

Unlike Th1 responses, a Th2 response emerges during the chronic phase of TB<sup>226</sup> and may contribute to disease persistence and risk of reactivation<sup>227</sup>. Th2 cytokines are generally considered to dampen the Th1 response and are associated with lack of protection<sup>228</sup>. Overexpression of IL-10 was found to be involved in reactivation of *Mtb* in a naturally reactivation-resistant C57BL/6 mouse strain and increased growth of BCG<sup>229</sup>. Wangoo, *et al.* <sup>230</sup> have demonstrated that adoptive transfer of Th2 cells caused more extensive lung lesions in mice compared to those that received Th1 cells.

By contrast, Hammaren, *et al.*<sup>231</sup> have demonstrated that the inability to induce a Th2 response seems to be associated with progressive *M. marinum* infection, at least in zebra fish, arguing against the well-established adverse effects of Th2 responses in controlling mycobacterial infection. A study by Dwivedi, *et al.*<sup>232</sup> provided evidence that virulent strains of *Mtb*, but not BCG or mutant-ESAT-6 or -RD1 strains, promote the secretion of IL-1 $\beta$  in DCs, which directs Th2 cell differentiation. These findings suggest that IL-1 $\beta$  production induced by virulent mycobacterial stains may facilitate disease progression by suppressing the protective Th1 response. However, both the presence and absence of Th2 associated responses in patients with active TB have been reported<sup>233</sup>.

## 1.1.8.2.1.1.3 Th17 & Th22 cells

The differentiation of IL-17 and IL-22 producing Th17 CD4<sup>+</sup> T cell subsets requires a set of transcription factors including STAT3, retinoic acid receptor related orphan receptor  $\gamma$  (ROR $\gamma$ ), ROR $\alpha$  and basic leucine zipper transcription factor (Batf)<sup>234</sup>. The role of Th17 cells in *Mtb* infection is complex and conflicting. Th17-mediated cytokine and chemokine production induces the recruitment of neutrophils and macrophages to the site of infection suggesting a role in protective immunity<sup>235</sup>. Conversely, Th17-driven granulocyte recruitment, including neutrophils, has been suggested to enhance TB severity<sup>236</sup>. A recent study by Perreau, *et al.* <sup>237</sup> supports the involvement of Th17 cells in the early stages of the immune response. BCG vaccination induces a Th17 cell population in the lungs to produce

chemokines (i.e., CXCL9 and CXCL10) that recruit IFN-γ producing Th1 cells thereby restricting the growth of the bacilli<sup>238</sup>. IL-17 deficient mice infected with the virulent *Mtb* strain HN878 demonstrated elevated levels of bacteria in the lungs and defective cell recruitment<sup>239</sup>, while IL-17 was dispensable for immunity to the laboratory strain H37Rv<sup>240</sup>. Scriba, *et al.* <sup>241</sup> have shown a protective role of IL-17 and IL-22 producing CD4<sup>+</sup> cells in *Mtb* infection while others<sup>242</sup> have seen no difference in IL-17 expression in both healthy controls and TB patients. However, many studies suggest the involvement of Th17 in TB pathogenesis. High levels of IL-17 were observed in TB patients with persistently elevated antigen load<sup>243</sup>.

In contrast to mice, humans have a distinct subset of T helper cells called Th22 which can be found at sites of infections and produce IL-22. Th22 cells also expressed granzymes and IL-13. Whilst ROR $\gamma$  act as a positive regulator, transcription factor T-bet negatively regulate the Th22 differentiation<sup>244</sup>. IL-22 plays an important role as an *Mtb* growth inhibitor. Dhiman *et al*<sup>245</sup> have shown that recombinant IL-22 (rIL-22) acts through enhanced phagolysomal fusion and increased expression of anti-microbial peptide calganulin A to inhibit *Mtb* growth in human monocyte-derived macrophages (MDMs). In addition, rIL-22-treated BMDMs had significantly reduced intracellular *Mtb* HN878 burden and increased TNF- $\alpha$  production<sup>246</sup>. Furthermore, rIL-22 treatment resulted in increased expression of anti-mycrobial Reg3 $\gamma$ , S100A8 proteins and Lcn2 in lung epithelial cell line, C10<sup>246</sup>. Interestingly, low levels of serum IL-22 was found in TB/T2D comorbid patients compared to TB patients<sup>247</sup>. In a more recent animal study on TB/T2D comorbidity, rIL-22 treatment prolonged the survival of *Mtb*-infected T2D mice and prevented neutrophil accumulation, excessive inflammation and epithelial cell damage<sup>176</sup>.

# 1.1.8.2.1.1.4 Treg cells

CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells ( $T_{reg}$ ) are widely recognised for their role in dampening down effector immune responses, which may facilitate persistence of the pathogen within host and possible disease reactivation. They may, however, protect the host from excessive inflammation<sup>248</sup>.  $T_{reg}$  cells can be induced in an antigen-TCR dependent or independent manner such as recognition of bacterial components via TLRs<sup>249</sup>.  $T_{reg}$  are known to suppress Th1mediated pro-inflammatory responses through multiple mechanisms, such as production of anti-inflammatory cytokines (IL-10 and transforming growth factor-beta; TGF- $\beta$ ) and expression of cytotoxic T lymphocyte antigen-4 (CTLA-4)<sup>250</sup>. Shafiani, *et al.* <sup>251</sup> have shown that *Mtb*-specific  $T_{reg}$  cells delay the priming of effector T cells, thus potently restricting protective immunity during the early stage of the disease rather than directly inhibiting the Th1 response. This is mediated by the interaction between *Mtb*-specific T<sub>reg</sub> cells and APCs, which downregulate co-stimulatory function through the engagement of CTLA-4 and CD80/CD86<sup>252</sup>. Strikingly, antigen-specific T<sub>reg</sub> cells have been shown to expand from pre-existing Foxp3<sup>+</sup> T<sub>reg</sub> cells during *Mtb* but not *Listeria monocytogenes* infections<sup>253</sup>. Delayed cell-mediated immunity allows bacteria to establish in the lungs <sup>254</sup>. Interestingly, Foxp3<sup>+</sup> T<sub>reg</sub> cells can also be found within granulomas<sup>255</sup>, suggesting a possible regulatory role during chronic *Mtb* infection. T<sub>reg</sub> cells also suppress the activation and proliferation of other subsets of T cells<sup>256</sup> and the depletion of T<sub>reg</sub> cells was associated with increased IFN- $\gamma$ -producing cells<sup>257</sup>. Despite their renowned role in immunosuppression, recent experimental findings indicate that T<sub>reg</sub> cells may facilitate memory formation through promoting antigen persistence<sup>258</sup>.

## 1.1.8.2.1.1.5 CD4<sup>+</sup> memory T cells

Immunological memory is the hallmark of adaptive and vaccine-induced immunity<sup>259</sup>. IL-7 promotes the transition of CD4<sup>+</sup> effector T cells to CD4<sup>+</sup> memory T cells and acts as the main survival cytokine for CD4<sup>+</sup> memory T cells<sup>260</sup>. Memory cells mount an immediate robust response to any threat during a subsequent antigen encounter. Different memory T cell phenotypes have been identified. Both CD45RA<sup>-</sup> CCR7<sup>+</sup> T central memory cells ( $T_{CM}$ ) and CD45RA<sup>-</sup> CCR7<sup>-</sup> T effector memory cells ( $T_{EM}$ ) produce effector cytokines.  $T_{CM}$  reside mainly in lymphoid tissues while  $T_{EM}$  migrate to multiple peripheral tissue sites during an infection. Recent animal studies have established the existence of a non-circulating subset of tissue resident T memory cells ( $T_{RM}$ )<sup>261,262</sup>.  $T_{CM}$  display a comparably higher proliferation rate upon antigen stimulation. Cattle experimentally infected with *M. bovis* have displayed  $T_{CM}$  being differentiated into effector phenotypes such as CD4<sup>+</sup> effector T cells and  $T_{EM}$ <sup>263</sup>.

Antigen-specific CD4<sup>+</sup> memory T cells have been found in animals previously exposed to *Mtb* or BCG<sup>264</sup>. CD69 expressing CD4<sup>+</sup> T<sub>RM</sub> cells in lungs demonstrate rapid recall function<sup>265</sup>. An elegant study by Perdomo, *et al.* <sup>266</sup> has shown that mucosal BCG vaccination can induce protective immunity against *Mtb* largely through *Mtb*-specific CD103<sup>+</sup>CD69<sup>+</sup> T<sub>RM</sub> cells in lung tissue and fluid. Cytokine producing CD4<sup>+</sup> memory T cells (especially IFN- $\gamma$ -producing ones) play an important role during TB infection<sup>205</sup>. Andersen, *et al.* <sup>267</sup> demonstrated that classical memory cells that express CD45RB<sup>low</sup>, CD44<sup>high</sup>, and L-selectin<sup>low</sup> revert into CD45RB<sup>high</sup> CD44<sup>low</sup> L-selectin<sup>high</sup> cells in the long-term protection against *Mtb* in mice. However, the precise nature of a protective role of CD4<sup>+</sup> memory T cells in TB remains to be defined.

#### 1.1.8.2.1.2 CD8<sup>+</sup> T cells

CD8<sup>+</sup> T cells are renowned for their role in cytotoxicity. Although most of the findings strongly support the dominant role of Th1 cell-mediated immunity in *Mtb* infections, recent advances in identification of *Mtb* proteins have provided researchers with the much-needed tools to explore *Mtb*-specific CD8<sup>+</sup> T cells and their function. Antigen-specific CD8<sup>+</sup> T cells have been identified in BCG-vaccinated individuals as well as in patients with active TB and LTBI. Several studies have revealed that CD8<sup>+</sup> T cells have a greater role during latency compared to the acute phase of the disease<sup>268</sup>. Non-classical CD1-restricted CD8<sup>+</sup> T cells also elicit protective immune response against mycobacterial infections<sup>269</sup>. By contrast, mice lacking TAP-1, but not CD1d had weakened immune responses to *Mtb*, demonstrating the superiority of classical MHC class I-restricted CD8<sup>+</sup> T cells in protective immunity compared to the CD1drestricted CD8<sup>+</sup> T cells<sup>196</sup>. Several key studies have demonstrated the protective role of CD8<sup>+</sup> T cells during virulent *Mtb* infections in mice<sup>206,270,271</sup>. Flynn, *et al.* <sup>270</sup> showed that mice deficient of  $\beta_2$ -microglobulin, failed to control the growth of bacteria following intravenous (i.v.) infection. Recipients of adoptively transferred CD8<sup>+</sup> T cells from immune animals have developed resistance against mycobacterial infections<sup>272,273</sup>. In contrast, Mogues, et al. <sup>274</sup> demonstrated that CD8<sup>+</sup> T cells are not essential for the control of the infection.

While IFN- $\gamma$  mediated protection to *Mtb* is mainly afforded by CD4<sup>+</sup> T cells, IFN- $\gamma$  production by *Mtb*-specific CD8<sup>+</sup> T cells has also been observed<sup>275,276</sup>. IFN- $\gamma$  can induce the activation of macrophages to be bactericidal mainly through triggering the production of reactive intermediates as well as upregulating the MHC class I molecules, making them susceptible for T cell mediated killing<sup>277</sup>. In addition CD8<sup>+</sup> T cells, similar to CD4<sup>+</sup> T cells induce apoptosis of infected macrophages via Fas-FasL pathway<sup>214</sup>. CD8<sup>+</sup> T cells are well-known to kill *Mtb* by disrupting the cell wall via the release of cytotoxic granules containing granulysin, in a perforin-dependent manner<sup>278-280</sup>. The presence of both cytotoxic and IFN-γ producing CD8<sup>+</sup> T cells in the lungs of mice has been evident during the acute phase of *Mtb* infection<sup>280</sup>. The same group later demonstrated a rapid mobilization of CD8<sup>+</sup> T cells to the lungs and their participation in immune memory during secondary *Mtb* infection<sup>281</sup>. Memory formation is critical for an effective vaccine. Perdomo, et al. <sup>266</sup> have experimentally shown that mucosal BCG vaccination induces the generation of airway-resident memory CD8<sup>+</sup>CD103<sup>+</sup>CD69<sup>+</sup>T<sub>RM</sub> cells that displayed prototypical T<sub>RM</sub> features. The authors concluded that T<sub>RM</sub> cells, mainly CD8<sup>+</sup> cells, are pivotal for the enhanced protection induced by mucosal BCG administration. T cell mediated immune response to *Mtb* is summarised in Figure 1-6.



Figure 1-6: Overview of the cell mediated immune response in TB.

Mycobacterial peptides are presented to CD4<sup>+</sup> T cells in the context of MHC class II while CD8<sup>+</sup> T cells requires antigens to be presented in MHC class I molecules. Activated CD4<sup>+</sup> effector T cells can differentiate in to various T cell subsets. Subsequent production of pro-inflammatory cytokines (IL-2, IFN- $\gamma$  and TNF- $\alpha$ ) by Th1 cells further activates T cells and macrophages. Activated CD8<sup>+</sup> cells also produce IFN- $\gamma$  and TNF- $\alpha$  and act as cytolytic T lymphocytes (CTL) by secreting perforin and granulysin. Memory T cells produce multiple proinflammatory cytokines. Th17 cells play a role in activating polymorphonuclear granulocytes. Anti-inflammatory cytokines produced by both Th2 and T<sub>reg</sub> cells usually down-regulate the Th1 response and may lead to granuloma caseation which facilitate the escape of *Mtb* from solid granuloma. Figure adapted from Kaufmann, *et al.* <sup>99</sup>.

## 1.1.8.2.2 Humoral immunity to Mtb

Protection against *Mtb* is largely thought to be driven by cell-mediated immune responses. However, a number of studies suggest a potential role of B cell-driven humoral immunity in controlling intracellular pathogens by interacting with T cell immunity<sup>282,283</sup>. Antigen recognition by surface immunoglobulin triggers phagocytosis. Internalised antigens are further processed and presented in MHC class II molecules, which can be recognised by antigenspecific CD4<sup>+</sup> T cells thus influencing T cell activation. This recognition also triggers B cell proliferation, differentiation and maturation<sup>284</sup>. Some microbes however are found to activate B cells in the absence of CD4<sup>+</sup> T cells<sup>285</sup>. Antibodies are generally known to play a role in clearing microbes by participating in opsonisation, complement fixation and antibodymediated cytotoxicity<sup>286</sup>. B cell deficient mice have increased mycobacterial load<sup>287</sup> and adoptive transfer of B cells contains the bacteria while resolving the inflammatory exacerbation<sup>288</sup>. The inhibitory Fcγ receptor IIB (FcγRIIB) downregulates T cell activation by hindering DC maturation and subsequent antigen presentation<sup>289</sup>. Mice lacking FcyRIIB exhibit enhanced lung Th1 responses with reduced immunopathology while stimulatory FcyRdefective mice are more susceptible to mycobacterial disease<sup>290,291</sup>. Furthermore, evidence from passive transfer, monoclonal therapeutics, cohort and vaccine studies has coalesced into a compelling argument for the importance of antibodies<sup>292</sup>. In addition, Lu, et al. <sup>293</sup> found that active and latent TB infections have distinct *Mtb*-specific humoral responses characterised by unique Ab Fc functional profiles, selective binding to FcyRIII, and distinct Ab glycosylation patterns. For an instance, differential glycosylation of Abs in LTBI has enhanced certain innate effector functions such as phagolysomal maturation, inflammasome activation, and most importantly, macrophage killing of intracellular *Mtb*. These observations support a protective role of antibodies in TB. Interactions between immune complexes and FcyR in protective immunity is well established. Improved disease outcome and enhanced survival has been reported in immune complex (monoclonal antibodies coated with bacilli) treated Mtb-infected mice<sup>294</sup>. Immune complex treatment has also increased the production of pro-inflammatory cytokines such as IL-12p40 by macrophages in infected mice lacking FcyRIIB<sup>290</sup>. Furthermore, treatment of *Mtb*-infected mice with a high dose of i.v. immunoglobulin demonstrated reduced bacterial loads in both lungs and spleen<sup>295</sup>. Moreover, B cells are found in the outer layer of TB granulomas<sup>296</sup> and disrupted granulomatous structures have been reported in B cell deficient mice<sup>288</sup>.

B cells produce a variety of cytokines. Differential cytokine expression by B effector 1 (Be1) and Be2 B cells influences the differentiation of naïve CD4<sup>+</sup> T cells into Th1 and Th2 subsets, respectively<sup>297</sup>. Heightened production of anti-inflammatory IL-10 has been observed in both B cell deficient<sup>288</sup> and Fc $\gamma$ R-defective mice<sup>290</sup>. Elevated levels of anti-inflammatory cytokines including IL-10 are thought to be due to a compensatory attempt at lowering excessive inflammation. However, the role of B cell-mediated production of IL-10 is not clear.

# 1.1.9 Latent tuberculosis infection and the role of the granuloma

Approximately 1.7 billion people are 'latently infected' with *Mtb*<sup>8</sup>, representing an enormous repository of potential TB reactivation. A single active TB patient is believed to be capable of infecting as many as 45 other people<sup>298</sup> posing a major hurdle to the global control of TB. LTBI is characterised by a continuous immune response to *Mtb* in healthy individuals without clinical symptoms of TB.

When both innate and adaptive responses fail to cope with the rapid bacterial growth and to eradicate the pathogen, immune cells and anti-inflammatory cytokines act around the infected macrophages to control dissemination by remodelling the site of infection into a cellular structure called granuloma or tubercle<sup>37,299</sup>. The formation of granulomas can take up to 2-3 weeks in mice<sup>206</sup> and these granulomas provide both the immunological and physical barrier to 'wall off'  $Mtb^{298}$ . An estimated 5-10% of latently infected patients are at risk of reactivating TB in their lifetime. The majority develop active disease within the first five years after the initial infection<sup>81</sup>. The immune-competency of the host and the strain virulence largely determines the risk of reactivating disease in LTBI individuals<sup>36</sup>. In addition, conditions such as HIV, diabetes, smoking, alcoholism, renal diseases, malignancies and immune suppressive treatments are among the major risk factors for reactivation<sup>300</sup>. Epidemiological data indicate that HIV-infected individuals<sup>301</sup> and people who receive TNF inhibitors<sup>302</sup> are at the highest risk of developing active disease from LTBI reactivation. A study by Lin, et al. <sup>303</sup> has shown that anti-TNF could disseminate acute and latent Mtb infection with normal granuloma structure in non-human primates. In mouse models<sup>304</sup> TNF neutralization results in granuloma breakdown. Structural deficiencies in granulomas has also been observed in TNF KO mice<sup>305</sup> indicating the importance of TNF in granuloma formation and integrity, at least in mice.

Unlike in mice, human TB granulomas are highly stratified structures with infected macrophages in the core, surrounded by macrophages, T cells, B cells, neutrophils, endothelial cells, NK cells and fibroblasts<sup>306,307</sup>. However, more human-like lung pathology with hypoxic necrotizing lung lesions were observed in *Nos2*-/- mice<sup>308</sup>. The centre of the granuloma is often necrotic and known as caseum<sup>309</sup>. Macrophages residing within the granuloma have been reported to undergo morphological changes. They can be fused into multinucleated giant cells<sup>310</sup> or differentiate into lipid-dense foam cells<sup>311</sup>, while mature macrophages transform into epithelioid cells<sup>312</sup>. IFN- $\gamma$  and TNF- $\alpha$  production by CD4<sup>+</sup> T cells at the site of infection activates macrophages followed by the induction of pro-inflammatory cytokine production, including TNF- $\alpha^{298,313}$ . TNF- $\alpha$ -dependent chemokine production plays a major role in governing cell recruitment, which is critical for the formation of granulomas<sup>314</sup>. *In vitro* experiments by Algood, *et al.* <sup>315</sup> demonstrated that the expression of certain chemokines such as CCL2, CCL5, CXCL9, CXCL10, CXCL11 during *Mtb* infection was at least partly dependent on TNF- $\alpha$ . The accumulation of chemokines forms a chemical gradient that induces the migration of immune cells from blood vessels and other areas in the lungs to the site of

infection<sup>316</sup>. In addition, DCs also recruited to the site of infection where T cells are further activated and differentiate into Th1 cells<sup>184</sup> (**Figure 1-7**).



Figure 1-7: Structure of a typical granuloma.

Microscopic (left) and schematic (right) images of "classical granuloma" consisting of a circular lesion with lymphocytes and macrophages surrounding a central region of developing necrosis. Figures adapted from Orme, *et al.* <sup>317</sup> and Ramakrishnan <sup>307</sup>.

Although the initial protection from *Mtb* in mice is largely, but not completely, mediated by  $CD4^+$  T cells through IFN- $\gamma$  production<sup>206</sup>, long term protection mainly depends on successful granuloma formation<sup>318</sup>. Saunders, *et al.* <sup>318</sup> have also observed a population of IFN- $\gamma$  producing NK cells being recruited to lungs in a CD4<sup>+</sup> T cell-independent manner during a later stage of the disease. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cells are present in the granuloma<sup>319</sup>. Mice with defective CD4<sup>+</sup> T cells show delayed or poor granulomatous response to mycobacterial infection<sup>206</sup>. Similarly, CD8<sup>+</sup> T cell deficient mice form functionally impaired granulomas<sup>270</sup> suggesting the importance of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells in granuloma formation and maintaining its structural integrity.

Interestingly, as previously mentioned, the virulence of *Mtb* strains may also define the morphological and functional characteristics of the granuloma. For instance, the migration of macrophages is believed to be largely governed by RD1-dependent signalling. RD1-deficient strains, such as BCG, demonstrate slower kinetics in host cell recruitment<sup>320</sup>. This RD1-dependent chemotactic effect is mediated through ESAT-6, which triggers the production of metalloproteinase 9 (MMP9)<sup>321</sup>. The role of MMP9 in macrophage recruitment during *Mtb* infection has been experimentally demonstrated in *Mmp9* KO mice where a reduced number of macrophages and poor granuloma formation was observed<sup>322</sup>.

Pro- and anti-inflammatory responses control the fate of *Mtb*-containing granulomas. Any immune disruption will compromise the structural integrity of the granuloma and promote the accumulation of caseum in its centre. The collapsing granuloma releases infectious bacilli to other parts of the lung as well as into the airways facilitating the spread of *Mtb* through productive cough. This is known as 'disease reactivation' in LTBI.

## 1.1.10 Immune evasion

Many consider *Mtb* to be the world's most successful pathogen<sup>323,324</sup>. It utilises different mechanisms to evade host resistance by interfering with innate and adaptive immune responses<sup>325</sup>, enabling the bacteria to persist and to establish either progressive or latent infection. Studies suggest that most *Mtb* survival strategies facilitate bacterial persistence within infected macrophages by disrupting host innate effector mechanisms<sup>326</sup> and by preventing the induction of cell mediated immune responses through delayed antigen presentation<sup>327</sup>.

PRR signalling is important for the uptake of *Mtb* and the induction of host innate immune responses. As previously discussed, TLRs are involved in recognition of various mycobacterial-derived antigens. However, in contrast to non-pathogenic mycobacteria, virulent *Mtb* strains release modified cell wall antigens, such as ManLAM, which confounds the capacity of LAM to initiate receptor signalling and production of inflammatory mediators<sup>328</sup>. Similarly, modified MDP and TDM from a *pcaA* mutant of *Mtb*, which are recognised by NOD2 and C-type lectin, respectively, induce significantly reduced levels of pro-inflammatory cytokines<sup>329</sup>.

Activated macrophages induce multiple anti-mycobacterial mechanisms, including phagolysosome fusion and respiratory burst. However, *Mtb* has evolved a number of refined mechanisms to arrest phagosome maturation and phagolysosome formation. As previously discussed, the arrest of phagosome maturation enables bacteria to persist within a non-acidified intracellular host compartment. At which stage phagosome maturation is arrested is not clear yet<sup>330</sup>. In synergy with Rab, SNARE proteins (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) and tethering molecules regulate membrane fusogenicity<sup>331</sup>. *Mtb*-derived ManLAM has been found to inhibit the recruitment of a tethering molecule, namely EEA1, thus preventing phagolysosomal fusion<sup>332</sup>. Virulent mycobacteria have also been reported to alter cellubervin, a SNARE protein, possibly causing phagosome maturation arrest<sup>333</sup>. In addition, other *in vitro* studies have shown that live BCG are associated with

coronin 1 (also known as TACO), a 43 kDa tryptophan–aspartic acid repeat actin-binding protein of the host<sup>334</sup>, suggesting that the retention of TACO by mycobacteria on the surface of phagosome may prevent the fusion process. Romagnoli, *et al.* <sup>335</sup> have reported that virulent *Mtb* could induce an autophagy block in an ESAT-6-dependent manner to increase its intracellular survival.

The role of RNS in protective immunity against *Mtb* is well-established. Several virulence proteins are known to intervene in the nitrosative stress such as AhpC<sup>39</sup>, which breaks down the peroxynitrite anion (ONOO<sup>-</sup>), a potent oxidant<sup>336</sup>. Similarly, products encoded by pathogenic *Mtb* genes,  $noxR1^{337}$  and  $noxR3^{338}$ , are essential for resistance to RNS. The *Mtb* encoded membrane associated protein Rv3671c is also important for maintaining intrabacterial pH by resisting phagolysosomal acidic concentrations<sup>339</sup>. Davis, *et al.* <sup>340</sup> have demonstrated that *Mtb* affects the scaffolding protein EBP50, which is involved in inducible NOS (iNOS) recruitment.

The adaptive immune response to Mtb is remarkably delayed, probably due to the slow migration of infected DCs to lymph nodes. This delay enables the bacterium to grow rapidly during the early stage of infection<sup>327</sup>. *Mtb* has developed sophisticated mechanisms to interfere with the cell mediated immune response predominantly by manipulating antigen processing and presentation via MHC class I and class II pathways<sup>325</sup>. Reduced expression of MHC class II molecules was identified in lung macrophages of mice following aerosol BCG infection<sup>341</sup>. Similarly, live, but not dead, Mtb were found to decrease MHC class II antigen processing within phagosomes during early infection<sup>342</sup>. The recognition of a 19-kDa lipoprotein by TLR2 disrupts the antigen processing of the MHC class II pathway<sup>343</sup> by reducing the expression of class II trans-activator (CIITA)<sup>344</sup>. In addition, intracellular sequestration of immature MHC class II molecules has been observed in Mtb-infected macrophages<sup>345</sup>. Furthermore, Mtbderived proteins such as PE PGRS family of proteins play a potential role in inhibiting ubiquitin-dependent proteosomal degradation, thus supressing MHC class I antigen generation<sup>346</sup>. PE PGRS47 was recently found to inhibit autophagy that is associated with altered calcium fluxes at both the plasma membrane and ER, potentially interfering with MHC class II presentation<sup>347</sup>.

*Mtb* lipid antigens are presented in CD1 molecules via cross-presentation, which depends on the ability of infected macrophages to trigger apoptosis<sup>194</sup>. However, virulent *Mtb* strains were found to upregulate the anti-apoptotic Bcl-2 family member, Mcl-1, which actively participates

in blocking the apoptosis process<sup>348</sup>, thus modulating CD1 antigen presentation. These evasion mechanisms facilitate bacterial persistence and survival within the host for an extended period while also providing potential targets for new therapeutic agents including vaccines.

#### 1.1.11 Microbiota and TB

Trillions of symbiotic microorganisms live on or within the human body and these diverse microbial communities are collectively known as the 'microbiota'<sup>349</sup>. Human gut constitutes a substantial microbial habitant and has even been considered to be an essential organ<sup>350</sup>. Compelling evidence suggest its involvement in basic biological processes, such as metabolism, immune modulation, mucosal barrier maintenance and major disease conditions. These include metabolic diseases, respiratory diseases, mental and physiological diseases, autoimmune diseases and infectious diseases, including pulmonary TB<sup>351</sup>. Interestingly, the microbiota has been shown to exert effects at distal sites across the body via the so called gutlung<sup>352</sup>, gut-brain<sup>353</sup> and gut-liver<sup>354</sup> axes. Although it was assumed for decades that the lung is a largely microbiota-free environment, healthy lungs are no-longer considered sterile<sup>355</sup>. The human respiratory system harbours a distinct microbiota and shows spatial variation where for an instance lower lung compartments have reduced bacterial biomass compared to upper compartments<sup>356</sup>. Factors such as antibiotic consumption, nutrition or infections can cause microbiota dysbiosis, thus influencing the host-defence and immunity which in turn contribute to disease exacerbation<sup>356</sup>. Moreover, a majority of TB risk factors, such as excessive alcohol consumption, smoking and diabetes have shown to affect the host-microbiota<sup>357</sup>.

*Mtb* infection itself significantly alters the composition of the gut microbiota in humans<sup>358</sup> and animals<sup>359</sup> and results in a more diverse lung microbiota with higher presence of opportunistic bacteria<sup>358,360-362</sup>. A study by Perry *et al* has found that *Helicobacter pylori* infection in humans and NHPs may contribute to the control of *Mtb* infection<sup>363</sup>. In addition, physiological concentration of Indole-propionic acid from gut Clostridia spp, led to a significant reduction in spleen *Mtb* loads suggesting a potential anti-tubercular activity<sup>364</sup>. On the other hand, antibiotic-mediated induction of dysbiosis in gut microbiota provokes early susceptibility to *Mtb* infection and facilitates bacterial dissemination in mice<sup>365,366</sup>. This appears to be associated with the suppression of Th1 immunity and reduced expression of IFN- $\gamma$  and TNF- $\alpha$  levels<sup>365,366</sup>. Intriguingly, these phenotypes could be reversed by microbial reconstitution via faecal transplantation, signifying the importance of gut microbiota in conferring resistance to *Mtb*<sup>365,366</sup>. Similarly, a strong correlation has been established between reduced gut microbial diversity and increased risk of allergic asthma in young  $age^{367,368}$  and respiratory infections such as pneumococcal pneumonia<sup>369</sup> and influenza A virus<sup>370</sup>. The latter two studies point towards an association between depletion of gut microbiota and impaired function of DCs and AMs; two important players in anti-TB innate immunity<sup>371</sup>. Antibiotic-treated mice show lower levels of NLRP3-dependent IL-1 $\beta$  and IL-18 secretion resulting in failure of DCs to migrate from lung tissues to mediastinal lymph nodes and diminished antigen presenting capacity following influenza A infection<sup>370</sup>. DCs from gut microbiota depleted mice were associated with lower expression levels of co-stimulatory CD80, CD86 and MHCII molecules<sup>370</sup>. A more recent study demonstrated that gut microbiota dysbiosis impairs the phenotype and cytokine response of Mincle<sup>+</sup> lung DCs followed by defective Th1 and Th17 immune responses, thus promoting *Mtb* survival<sup>372</sup>. In addition, AMs derived from gut-microbiota depleted mice were had a diminished phagocytic capacity and low TNF- $\alpha$  levels upon *Streptococcus pneumoniae ex vivo* infection<sup>369</sup>.

T2D is a progressive metabolic disease<sup>373</sup> and the changes in intestinal microbiota composition have been linked to gut permeability resulting in metabolic endotoxemia which leads to chronic inflammation and insulin resistance seen in T2D<sup>374,375</sup> (discussed setensivley in section 1.3). Multiple studies have shown increased Firmicutes and reduced Bacteroidetes in different animal models of diabetes; such as mice fed with high-fat diet<sup>376,377</sup>, genetically obese *leptin*deficient *ob/ob* mice<sup>378</sup> and NOD mice<sup>379</sup>. The elevated Firmicutes/ Bacteroidetes ratio was also seen in obese people with insulin resistance<sup>380</sup> and T2D patients<sup>381,382</sup> compared to healthy individuals. Contradicting findings with reverse tendencies have also been reported<sup>383,384</sup>. How an altered intestinal microbiota in T2D, influence anti-TB immunity is not yet fully comprehended. However, it has been suggested that translocation of immune cells, bacteria and bacterial metabolites such as short chain fatty acids (SCFAs) from intestine to lung via the blood circulation could shape the immune responses<sup>352,385,386</sup>. SFCAs are known to induce antiinflammatory IL-10, tolerogenic T cell profiles in humans<sup>387</sup> and particularly butyrate which modulates mucosal immune responses acting mainly on innate immune cells, thereby suppressing the activation, differentiation and recruitment of neutrophils, macrophages and DCs<sup>388</sup>. Human PBMCs treated with butyrate demonstrate a significantly reduced production of *Mtb*-induced pro-inflammatory cytokines; IL-1β, TNF-α and IL-17A and increased IL-10<sup>389</sup> further supporting the correlation between increased butyrate levels and dampened anti-TB immune response. In a recent study, Nastasi and colleagues have shown that butyrate inhibit the activation and expansion antigen-specific TNF- $\alpha$  and IFN- $\gamma$  producing CD8<sup>+</sup> T cells by affecting the antigen-presenting cells<sup>390</sup>. On the contrary, beneficial roles of butyrate, such as promoting memory potential of activated CD8<sup>+</sup> T cells have also been reported<sup>391</sup>. Furthermore, butyrate treatment strongly reduced the secretion of IL-12p70 accompanied with reduced expression of co-stimulatory molecules CD40, CD80, CD83, CD86, MHC-I and MHC-II<sup>390</sup>. Similarly, butyrate suppressed LPS-induced activation of human DCs and promoted IL-10-secreting T regulatory cells<sup>392</sup>. Taken together these evidences support the notion that altered microbiota along with other factors associated with T2D renders APCs futile thus leading to increased susceptibility to TB. More importantly, based on the evidence presented above, the microbiota could also be a potential therapeutic target in the context of TB/T2D and other comorbidities.

## **1.2 TB/ HIV comorbidity**

HIV is a lentivirus belonging to the family of retroviridae and can be classified into HIV-I & -II based on distinct genetic characteristics<sup>393</sup>. HIV-1 causes acquired immunodeficiency syndrome (AIDS), an immune dysfunction mainly involving the gradual depletion of CD4<sup>+</sup> T cells which leads to the development of fatal opportunistic infections if untreated. In 2017 there were approximately 36.9 million people worldwide living with HIV/AIDS (HIV.gov 2018). HIV co-infection is the most potent risk factor for TB. Not only does it lead to increased susceptibility to active TB infections, but also enhances the rapid progression of the disease in HIV-patients which is associated with increased morbidity and mortality<sup>13</sup>. Furthermore, while 5-10% of non-HIV LTBI patients are at risk of reactivating TB in their lifetime, HIV coinfection increases the risk of reactivation from LTBI by 10-fold<sup>394</sup>. In 2017, there were 1.14 million active TB cases and ~300,000 TB deaths among HIV-1 positive individuals (WHO, 2018).

## 1.2.1 Pathophysiology of HIV

Sexual transmission represents the most common route of HIV infection globally<sup>395</sup>. Among different hypothesises as to how HIV causes AIDS, a considerable progress has been made so far over the last few decades to identify the mechanisms that the virus uses to gain access to host cells, which results in progressive loss of CD4<sup>+</sup> T cells.

Initially it was discovered that the HIV envelope (Env) protein attaches to the host cell, binding to its primary surface receptor CD4<sup>396</sup>. However, this did not explain as to how the virus infects antigen presenting cells, such as macrophages and dendritic cells<sup>397</sup>. This led to the discovery

of two chemokine receptors; CCR5 and CXCR4 as essential co-receptors for HIV-1 entry<sup>398</sup>. Initial Env-CD4 binding causes conformational changes in Env, allowing co-receptor molecules to engage in the binding process followed by membrane fusion and subsequent delivery of viral content into the cytoplasm of host cells<sup>399</sup>. Moreover, HIV-1 strains are generally classified based on their co-receptor usage. Strains that use CXCR4 and CCR5 are termed X4 HIV and R5 HIV, respectively, and those that use both co-receptors are known as R5X4 HIV<sup>400</sup>. X4 HIV; also known as T-tropic HIV infects T lymphocytes and T cell lines primarily through CXCR4 while R5 HIV or M-tropic HIV uses CCR5 to infect monocytes, macrophages and T lymphocytes, but not T cell lines. R5X4 or dual-tropic HIV strains utilise CCR5 and CXCR4 to gain entry into macrophages and T cell lines, respectively. Interestingly, R5X4 or dual tropic HIV strains can also utilise combinations of co-receptors to gain access to many cell types including blood and tissue macrophages, dendritic cells and T lymphocytes due to the co-expression of CXCR4 and CCR5<sup>401</sup>.

Once viral particles are released into the host cell, particularly into activated CD4<sup>+</sup> T cells, HIV-1 integrates into the host DNA, which leads to viral replication. It has been found that activated T cells provide optimal conditions for rapid and efficient viral replication. In contrast, quiescent CD4<sup>+</sup> T cells act as a latent viral reservoir by disrupting the viral replication at the transcriptional level thus permitting the establishment of provirus<sup>402</sup>. In addition to T cells, macrophages and dendritic cells also function as an important cellular reservoir which supports the survival of the virus by resisting immune and antiretroviral pressure. Dendritic cells in particular, can either be infected directly or recognise and capture virions using surface bound DC-SIGN<sup>403</sup>. The latter mechanism can also relay signals to T cells that promote their ability to sustain viral replication.

There are several mechanisms that contribute to viral cytopathogenicity which result in T cell depletion. Studies have shown that the fusogenicity or fusogenic capacity of viral envelope protein correlates with viral cytopathicity *in vitro*<sup>404</sup> as well as with the depletion of CD4<sup>+</sup> T cells *in vivo*<sup>405</sup>. In addition, it is believed that other viral proteins such as Vpr contribute to the direct killing of infected cells by promoting apoptosis, while accessory protein Nef which upregulates FasL contributes to bystander apoptosis of Fas-expressing activated HIV-1-specific CD4<sup>+</sup> T cells and CTLs<sup>402</sup>.

#### 1.2.2 *Mtb*/HIV co-infection

As previously described, chronic T cell activation and progressive loss of CD4<sup>+</sup> T cells is a major hallmark of HIV infection, and T cell-mediated immunity is also crucial for protection against TB. Therefore, increased risk of TB and bacterial dissemination strongly correlates with the depletion of *Mtb*-reactive CD4<sup>+</sup> T cells which produce pro-inflammatory cytokines such as TNF, IFN- $\gamma$  and IL-2 in HIV-1 infected individuals<sup>406</sup>. Transcriptional profiling of cells entering the tuberculin skin test site revealed that CD4<sub>+</sub> T cell recruitment and subsequent IFN- $\gamma$  production were significantly reduced in HIV-1/*Mtb* co-infected patients<sup>407</sup>. Furthermore, findings from animal studies suggest that in addition to the impact on Th1, Th17, Th22 T cells, the recruitment of phagocytic cells such as macrophages may also be affected in this comorbidity<sup>408</sup>. Innate-like CD8<sup>+</sup> MAIT cells are known to recognise *Mtb* metabolites and their depletion in HIV-1 infection may also affect the host immune response to *Mtb*<sup>279</sup>.

HIV-1 accessory protein Nef was found to inhibit the recruitment of endosomes by interacting with adaptor protein-1 (AP-1) thus inhibiting phagocytosis in macrophages<sup>409</sup>. In line with these findings, impaired phagocytosis has been observed in HIV-1-infected alveolar macrophages *ex vivo*<sup>410</sup> and is associated with increased *Mtb* growth in co-infected macrophages<sup>411</sup>. Nef was also found to inhibit TNF response against *Mtb*, thus restricting apoptosis of infected macrophages which in turn permits the excessive intracellular growth of  $Mtb^{412}$ . However, more recent findings from live-cell imaging suggest that cell death is associated with *Mtb* growth due to the dissemination rather than restriction<sup>413</sup>.

It is unclear how HIV-1 interrupts the innate immune response to *Mtb*. However, HIV-1 accessory proteins Nef, Vpu and Ver have been found to inhibit intracellular signalling pathways. Attenuated IL-10 expression in HIV-1-infected cells may also cause exaggerated pro-inflammatory responses to *Mtb* infection<sup>414</sup>. Interestingly, the immune response to *Mtb* increases HIV-1 transcription through binding to several host transcription factors such as C/EBP, nuclear factor kappa B (NF- $\kappa$ B) and nuclear factor of activated T cells 5 (NFAT5)<sup>415</sup>.

Reactivation of LTBI is mainly driven by the disruption of *Mtb*-containing granulomas which facilitates bacterial dissemination. T cells, among other immune cells, play a central role in maintaining the structural integrity of granulomas thereby containing *Mtb* for prolonged periods after infection. However, HIV-1 preferentially depletes *Mtb* antigen-specific CD4<sup>+</sup> T cells<sup>416</sup> thus changing the formation, organisation, caseation and cellular population of

granulomas<sup>417</sup>. Collectively, these findings suggest that HIV-1 ultimately promotes *Mtb* replication and dissemination in co-infected individuals rendering the global control of TB even more challenging.

## 1.2.3 Animal models of *Mtb*/HIV co-infection

It is important to study HIV in-depth to decipher the underlying immunological defects which could potentially lead to increased susceptibility of TB in comorbid patients. Animal models are therefore mandatory for HIV studies as certain experimental interventions, treatments (cure) cannot be directly tested on humans without major risks. Animal models not only provide the *in vivo* systems to study multiple anatomic reservoirs but also permit interventions aimed at depleting various arms or specific immune cell populations of the immune system. However, HIV's restriction to human cells has been a major limitation for HIV studies in animal models<sup>418</sup>. Simian immunodeficiency virus (SIV) closely resembles and shares key pathogenic features of HIV infection in non-human primates (NHP), but NHP studies are associated with numerous ethical, financial and logistical limitations<sup>419,420</sup>. Regardless, NHP models have proven useful in LTBI reactivation studies with several latency reversal agents (LRAs)<sup>421</sup>. Initial attempts to infect small animals with HIV-1 were unsuccessful due to the lack of proteins that are necessary for HIV-1 infection<sup>422</sup>. Although, transgenic animal models did overcome this obstacle, it was later found that cells from these mice actually express inhibitory proteins that might block HIV-1 replication<sup>423</sup>. As technology has advanced, humanized mice have been generated by engrafting human tissues or hematopoietic progenitor cells in genetically immunocompromised mice<sup>424</sup>. Similarly, a modified HIV strain; EcoHIV was developed by replacing gp120 in HIV-1 with gp80 from murine leukaemia virus allowing HIV-1 to infect rodent cells and overcome the HIV-1's human cell restriction<sup>424</sup>. Scid-hu-*Thy/Liv*, scid-*hu-PBL*, *NOD scid Il2rg<sup>-/-</sup>* and *Rag2<sup>-/-</sup> Il2rg<sup>-/-</sup>* are among the most frequently used humanized mice in HIV-1 research<sup>418</sup>. However, these animal models present with limited potential as a reliable, affordable and traceable animal model to study HIV/ Mtb co-infection.

In order to study TB/HIV comorbidity in the context of LTBI reactivation, I used a murine TB ear dermis infection model (intradermally infected) which reflects several aspects of LTBI reactivation in humans following the loss of CD4<sup>+</sup> T cells as it occurs in HIV-1-infected individuals<sup>425</sup>. In contrast to the CD4- and MHCII-deficient mice which do not comprise all latent aspects of LTBI but rather presented with exacerbated disease compared to control C57BL/6 mice<sup>426</sup>, this model mimics the LTBI reactivation in HIV-1 by containing *Mtb* within
local draining LNs until CD4<sup>+</sup> T cell depletion. It has been speculated that TB has characteristic features of a lymphatic disease<sup>427</sup> and findings from recent NHP studies also provide evidence of prolonged *Mtb* persistence in LNs during TB infection<sup>428,429</sup> warranting further exploration of this ear dermis infection model to study LTBI reactivation.

# **1.3 TB/ T2D comorbidity**

#### **1.3.1** Diabetes mellitus

DM is one of humankind's most ancient diseases, with a written history of approximately 3,000 years<sup>430</sup>. It represents a group of complex metabolic conditions characterized by hyperglycaemia due to the impairments in insulin production, insulin action, or both. Chronic hyperglycaemia is often associated with, but not limited to, ketoacidosis, strokes and multiple organ dysfunctions and failures, such as in eyes, kidneys, heart, and blood vessels<sup>431</sup>. Most DM cases fall into two main categories: Type I and Type II. In addition, onset or first recognition of any glucose intolerance during pregnancy is defined as gestational diabetes mellitus (GDM). Type I diabetes (T1D) or juvenile-onset diabetes results from an autoimmune destruction of  $\beta$ -cells of islets of Langerhans in the pancreas, leading to absolute insulin deficiency<sup>431</sup>. In addition, T1D has a strong association with various susceptibility genes (*HLA*, insulin gene, *PTPN22*, *IL2Ra*, and *CTLA4*) and environmental triggers<sup>432</sup>. T1D accounts for only 5-10% of total diabetes cases. T2D or adult-onset diabetes, which is the most common form of diabetes, accounts for more than 90% of total diabetes incidences<sup>431</sup>. Rather than an absolute insulin deficiency and/or insulin resistance<sup>433</sup> and do not typically require exogenous insulin to survive<sup>434</sup>.

### 1.3.1.1 Epidemiology

The prevalence of diabetes is rapidly increasing across the globe, mostly due to pronounced changes in environment and human lifestyle accompanied by a rapid increase in global population<sup>434,435</sup>. Striking figures by the International Diabetes Federation (IDF) revealed that about 80% of those with diabetes currently live in low and middle income countries, regardless of diabetes being widely recognised in high income countries<sup>436</sup>. Approximately 463 million people were estimated to have diabetes in 2018 and the numbers are expected to escalate to 700 million by 2045<sup>436</sup>. The unprecedented increase in prevalence of diabetes presents a great threat to economies and public health systems world-wide. For an instance, rapid emergence of non-communicable diseases such as T2D in Sub-Saharan African (SSA) countries which

already struggle with communicable diseases such as AIDS, malaria and TB adds an extra burden to the public health systems and to the people who live in this region<sup>437</sup>.

#### 1.3.1.2 Risk Factors

Development of T2D depends on many lifestyle, environmental and genetic factors<sup>438</sup>. Obesity, diet, reduced physical activities, sedentary lifestyle, smoking and alcoholism are among the well-described lifestyle factors in T2D<sup>433,439,440</sup>, while overweight and obesity (body-mass index; BMI  $\geq$ 25 kg/m<sup>2</sup>) affect most of the adult population in high-income countries<sup>441</sup>. Interestingly, the prevalence of obesity in Asia is relatively low and does not directly correlate with the increased rates of T2D<sup>442</sup>. In synergy with overweight and lower physical activities, high calorie food intake significantly increases the risk of developing T2D<sup>443</sup>. Moreover, smoking<sup>444</sup>, high alcohol intake<sup>445</sup> and host genetics (i.e., *GLUT4*, *RBP4*, *PEPCK* and *KCNJ11*) are strongly associated with disease pathogenicity<sup>446,447</sup>.

# 1.3.1.3 Pathophysiology of T2D

In normal physiology, glucose homeostasis is maintained by two potent regulatory hormones: glucagon and insulin, secreted by  $\alpha$ - and  $\beta$ -cells of the pancreas, respectively. Glucagon promotes glycogenolysis (breakdown of glycogen to produce glucose in tissues) and gluconeogenesis (generation of glucose from triglyceride (TG), lactate and amino acids) during fasting and intense physical activities while insulin promotes the deposition of glucose as glycogen in liver (glycogenesis). Insulin may also inhibit the production of glucagon from  $\alpha$ -cells<sup>448</sup>. The complex pathophysiology of T2D is largely driven by insulin resistance and impaired production of insulin due to gradual fall in  $\beta$ -cell function<sup>438</sup> results in decreased glucose transport to storage sites.

The exact mechanisms underlying how insulin resistance leads to T2D are not completely understood. Some believe the inability of insulin target tissues such as skeletal muscles to respond to insulin properly results in obesity<sup>449</sup>, while the reverse is proven to be true in many cases<sup>450</sup>. Insulin signalling is triggered by the binding of insulin to  $\alpha$  subunit of insulin receptor (IR) which ultimately leads to the translocation of glucose transporter 4 (GLUT4)<sup>449,451,452</sup>, an insulin dependent glucose transporter that uptakes glucose from the circulation into cells, thus regulating glucose homeostasis<sup>453</sup>. Interestingly, reduced expression of insulin receptor signalling pathway components such as GLUT4<sup>454</sup> and phosphoinositide 3-kinase (PI3K)<sup>455</sup> have been reported in T2D patients. Impaired phosphorylation of Akt2<sup>456</sup> and AS160 in

adipocytes as well as defective Rho-kinase (ROCK)<sup>449</sup> activity have also been reported in T2D patients. Moreover, animal studies have demonstrated that PKB/Akt2<sup>457</sup> but not Akt1/PKB isoform<sup>458</sup> is indispensable for glucose homeostasis and the development of a T2D phenotype, supporting the importance of signalling pathways in IR-mediated GLUT translocation. In addition to the known genetic and environmental factors, glucolipotoxicity, inflammatory mediators, mitochondrial dysfunction and endoplasmic reticulum (ER) stress could cause insulin resistance<sup>459</sup> via phosphorylation of signalling molecules mediated by serine and threonine (Ser/Thr) kinases, but not tyrosine kinase<sup>451</sup>.

During insulin resistance,  $\beta$ -cells of islets increase insulin secretion through enhanced  $\beta$ -cell function and increased  $\beta$ -cell mass<sup>460</sup>. However, the underlying mechanisms of  $\beta$ -cell compensation are not well defined. Reduced  $\beta$ -cell numbers are associated with T2D<sup>461</sup> and apoptosis may be the cause of the  $\beta$ -cell loss<sup>462</sup>. In addition to glucose, both glucagon-like peptide 1 (GLP-1) and free fatty acids (FFA) are known to stimulate  $\beta$ -cell compensation mechanisms<sup>460</sup>. Defective mitochondrial function, TG/FFA cycling, Adenosine monophosphate-activated protein kinase/malonyl-CoA signalling and ER stress play a substantial role in progressive  $\beta$ -cell damage and dysfunction<sup>460</sup>. The underlying molecular mechanisms have been extensively reviewed by Muoio, *et al.* <sup>463</sup>.

The role of inflammation in insulin sensitivity and  $\beta$ -cell dysfunction is well described<sup>464</sup>, and altered expression of cytokines, chemokines and different immune cell populations in adipose tissues and liver have been reported<sup>438,465</sup>. Significantly higher levels of inflammatory mediators such as IL-1 $\beta$ <sup>466</sup>, IL-6 and C-reactive proteins<sup>467</sup> are suggestive of T2D and reflect the activation of immune cells. Rapid expansion of adipocytes to cope with storing excess lipids in T2D leads to adipocyte cell death due to hypoxia, which triggers the upregulation of pro-inflammatory cytokines and chemokines through NF- $\kappa$ B and JUN N-terminal kinase (JNK) pathways<sup>465</sup>.

The role of TNF- $\alpha$  in tissue inflammation in T2D is well-known<sup>468</sup>. Hyperglycaemia-induced IL-6 production has displayed both detrimental<sup>469,470</sup> and beneficial roles<sup>471</sup> in the development of insulin resistance. IL-1 $\beta$ -driven upregulation of pro-apoptotic receptor FAS expression mediates  $\beta$ -cell apoptosis<sup>472</sup>, thus affecting  $\beta$ -cell secretory function. Additionally, IL-1 $\beta$  expression can be triggered by the direct binding of thioredoxin-interacting protein (TXNIP) to NLPR3 inflammasomes in a glucose- and ROS-sensitive manner<sup>473</sup> as well as by islet

amyloid polypeptide (IAPP)<sup>465</sup>. Abundant expression of IL-1R1 on  $\beta$ -cells could lead to IL-1 $\beta$  auto-inflammation<sup>474</sup>. In addition to cytokines, adipocytes of mice fed with a high-fat diet have produced elevated levels of chemokines such as CCL2, CCL3, CCL6, CCL7, CCL8 and CCL9<sup>475</sup>. The production of chemokines in islet cells is triggered by FFA-TLR (TLR2 or TLR4) or IL-1 $\beta$  signalling, which promotes tissue infiltration of macrophages, mast cells, CD4<sup>+</sup> Th1 and CD8<sup>+</sup> T cells<sup>465</sup>, demonstrating the inflammatory nature of T2D.

In addition, hyperglycaemia-induced ROS generation and inflammatory cytokine production often lead to many diabetic complications, such as atherosclerosis, retinopathy, renal failure and nerve damage<sup>476,477</sup>. Despite the elevated levels of inflammatory mediators observed, impaired recognition of PAMPs followed by defective phagocytosis and reduced levels of pathogen-stimulated pro-inflammatory cytokines, and oxidative mediators are linked to increased susceptibility to infections in diabetes<sup>478</sup>.

# **1.3.1.4** Diabetes treatments

T2D is mainly treated by oral and injectable drugs. New therapeutic approaches have emerged with the rapid increase in T2D prevalence over the past few decades<sup>438</sup>. Currently available antidiabetic drugs and their mode of actions are summarised in **Table 1-3**.

	Class of drug	Examples*	Mode of action	<b>Reference</b> <sup>†</sup>
Oral drugs	Second-generation sulfonylurea antidiabetics	Glibenclamide Gliclazide Glimepiride Glipizide	Inhibition of ATP-sensitive K-channels in $\beta$ cell	479
	Biguanide antidiabetics	Metformin	Downregulation of hepatic gluconeogenesis through transient inhibition of the mitochondrial respiratory-chain complex 1	480
	Thiazolidinedione antidiabetics	Pioglitazone Rosiglitazone	Activation of the gamma isoform of the peroxisome proliferator-activated receptor (PPAR-γ)	481
	α-glucosidase inhibitors	Acarbose Miglitol Voglibose	Reduction of glucose absorption rate by delaying the degradation of carbohydrates through inhibition of intestinal $\alpha$ - glucosidases	482
	DPP4 inhibitors	Alogliptin Linagliptin Saxagliptin Sitagliptin Vildagliptin	Inhibition of the degradation of incretins, glucagon-like peptide- 1 (GLP-1) and glucose- dependent insulinotropic peptide (GIP)	483

Table 1-3: Current anti-diabetic drugs

	SGLT2 inhibitors	Canagliflozin Dapagliflozin	Inhibition of SGLT2 in proximal convoluted tubule (PCT), to prevent reabsorption of glucose and facilitate its excretion in urine	484
	Glinides	Nateglinide Repaglinide	Inhibition of ATP-sensitive K- channels in β cell	485
Injectable drugs	GLP-1 receptor agonists	Exenatide Liraglutide Lixisenatide	Enhanced insulin production through $\beta$ cell proliferation and anti-apoptosis	486
	Rapid-acting and short- acting insulin	Soluble insulin Insulin aspart Insulin glulisine Insulin lispro Insulin zinc– amorphous	Inhibition of hepatic	487
	Intermediate-acting insulin	Isophane insulin Insulin zinc	biosynthesis of glucose	
	Long-acting insulin	Insulin zinc– crystalline Insulin detemir Insulin glargine		

\* as reviewed by Olokoba, et al. 433 and Kahn, et al. 438. † References for mode of action

#### 1.3.2 The double burden of TB/T2D

The elevated incidence of comorbid immunosuppressing conditions has been associated with high risk of intracellular bacterial infections and their complications<sup>488</sup>. HIV co-infection is the most potent risk factor for TB<sup>13</sup>. The global epidemic of diabetes, particularly type 2, due to rapid urbanization, high calorie diet, and changes in lifestyles (lower physical activity)<sup>441</sup>, has influenced the development of TB<sup>489</sup>, thus adversely affecting the control of the disease. The association between DM and TB has been recognised for centuries<sup>490</sup>. Although TB has been a disease of the poor, increased incidence of diabetes has meant that the affected population has a wider demographic spread<sup>491</sup>. Interestingly, comorbidity is significantly higher in wealthy urban TB populations than rural populations (14.0% vs 10.6%)<sup>488</sup>. Furthermore, increased risk of reactivating active TB from LTBI, presentation of more severe clinical outcomes and higher mortality rates are associated with TB-DM comorbid patients<sup>492</sup>.

# 1.3.2.1 Epidemiology

The global prevalence of diabetes is expected to escalate to 700 million by 2046<sup>436</sup> and up to 70% of diabetics currently live in TB endemic countries<sup>17</sup>. The top 22 countries with the highest TB burden had a diabetic population of 2.3% - 13.8% of their general population<sup>7</sup>. A retrospective study by Bridson, *et al.* <sup>493</sup> demonstrated a higher representation of diabetes in the Australian TB population, suggesting a strong association between DM and pulmonary TB, irrespective of TB endemicity. A similar association was observed in a population-based cohort

study conducted in the UK<sup>494</sup>, further confirming the enhanced risk of developing TB in diabetes.

Asia suffers from both TB and diabetes. India has the highest TB and second highest diabetes burden in the world, with an estimated 2.69 million TB cases <sup>8</sup> and nearly 70 million cases of diabetes<sup>436</sup>. Among those, 14.8% of TB cases could be attributed to diabetes<sup>495</sup>. The prevalence of diabetes (13.9%) and impaired fasting glucose (IFG) (8.9%) were noticeable among 110 newly diagnosed TB patients within a tribal population in Odisha, India<sup>496</sup>. However, in a large cohort study in India, almost half of the study TB population were either diabetic or pre-diabetic<sup>495</sup>. A recent community-based study in Bangladesh revealed that 28.3% of the TB population were either diabetic or pre-diabetic<sup>497</sup>. In a large cohort of 8886 Chinese TB patients, 1090 (12.4%) and 575 (7.8%) patients were found to be diabetic and IFG, respectively<sup>498</sup>. Indonesia, a tropical low-middle income country with the third highest TB burden in the world<sup>8</sup>, has the seventh highest number of diabetics<sup>436</sup>, globally confirming the positive correlation between TB and DM.

DM-TB comorbidity was also observed in about 12.3% of 4000 confirmed Nigerian TB cases during an observational study<sup>499</sup>. The higher incidence of diabetes has been reported in patients hospitalised for TB in the USA<sup>500,501</sup>. Hispanic and Asian/Pacific islanders with diabetes had increased odds of active TB disease compared to white and black patients, suggesting a possible demographic link between DM-TB comorbidity. TB was also common among diabetics in Papua New Guinea<sup>502</sup>. Additionally, Dooley, *et al.* <sup>489</sup> have reviewed a number of studies (1952-2008) on 'diabetes as a risk factor for TB' where the authors noticed a poor response to anti-TB drugs in some populations.

Recent large meta-analyses demonstrated that individuals with diabetes have a threefold risk of developing TB while more than 16% of TB patients have comorbid diabetes<sup>19,20</sup>. Therefore, there is an urgent need to understand the basic immunological mechanisms that contribute to the high susceptibility of diabetic patients developing active *Mtb* infection.

# 1.3.2.2 Impaired anti-TB immunity in diabetes

Several studies have revealed a strong correlation between elevated glycosylated haemoglobin (HbA<sub>1C</sub>) levels (chronic hyperglycaemia) and TB susceptibility<sup>503</sup>. This correlation may be linked to some of the metabolic pathways that are being affected by T2D.

Macrophages are the main effector cells involved in killing Mtb. Saiki, et al. 504 observed impaired phagocytic activity in macrophages in streptozotocin- (STZ-) induced diabetic mice that had a significantly lower resistance to Mtb compared to non-diabetic mice. Yamashiro, et al. <sup>505</sup> suggested that the reduced production of NO and Th1 cytokines are the leading cause of a defective immune response seen in diabetic mice. However, insulin treatment improved the Th1 cytokine levels and resulted in reduced organ bacterial loads. Further investigations by Martens, et al. 506 revealed that higher TB susceptibility was associated with STZ-induced chronic, but not acute diabetes. In addition, higher Th1 pro-inflammatory cytokines were also observed on STZ-treated mice. In vitro infection with the Mtb reference strain H37Rv has shown that hyperglycaemia alone does not significantly alter the phagocytic activity of macrophages<sup>507</sup>, which is in line with findings by Martens, et al. <sup>506</sup>. Elevated production of ROS and pro-inflammatory cytokines; IL-1 $\beta$  and TNF- $\alpha$ , have been reported in both diabetic rats<sup>508</sup> and TB-T2D patients<sup>247</sup>. Intriguingly, baseline ROS levels were also high in T2D patients<sup>509</sup> due to the inflammatory nature of the disease. These findings however contradict the previously reported reduced protective Th1 cytokine levels<sup>510</sup> and impaired phagocytic activities<sup>511</sup> in TB patients with diabetes. Reduced expression of anti-microbial peptides such as cathelicidin LL-37, human  $\beta$ -defensin (HBD)–2, HBD-3 and HNP has also been reported<sup>512</sup>. An interesting study by Chao, et al. <sup>513</sup> demonstrated that increased adipokines, specifically, resistin, may suppress the production of IL-1 $\beta$  through inflammasome activation by inhibiting the production of ROS in immune cells, resulting in ineffective Mtb control. Similarly, increased susceptibility and defective macrophage function was also observed in Burkholderia pseudomallei infected diabetic mice<sup>514</sup>. More recently, Martinez, et al. <sup>515</sup> have shown that impaired recognition of *Mtb* by alveolar macrophages of diabetic mice is mediated by reduced expression of CD14 and macrophage receptors with collagenous structure (MARCO) that play a role in recognition of TDM. The authors have also demonstrated that defective phagocytic activity of alveolar macrophages is RAGE (receptor for advanced glycation end products) dependent, linking it to the hyperglycaemia-induced AGE production. Interestingly, Ilyas, et al. <sup>516</sup> have found that high glucose levels disrupt the binding of c-type lectin and DC-SIGN to high-mannose glycoproteins and fucosylated ligand, respectively. In addition, the lectin pathway of complement activation was also abrogated. The authors suggested that high glucose levels in diabetes may alter the recognition of carbohydrate antigens via competitive inhibition leading to dysregulated immune responses against pathogens. Similarly, a significantly lower Mtb association with monocytes was determined in T2D patients. However, heat inactivation of serum reduced *Mtb*-monocyte interactions in both T2D and control groups to a similar level indicating possible deficiency in the complement pathway in T2D<sup>517</sup>. *Mycobacterium fortuitum* infected mice in a diet induced murine model of T2D (DIM2TD) had significantly higher bacterial loads, impaired bacterial internalisation and killing by both peritoneal and alveolar macrophages as well as an altered cytokine profile, indicating defective immune response against *M. fortuitum* infection<sup>518</sup>. Impaired neutrophil function is also associated with increased susceptibility to infections in diabetes<sup>519</sup>, which is thought to be mediated by RAGE<sup>520</sup>. Intracellular GSH levels play a significant role in augmenting NK cell activation and function<sup>162</sup> and GSH deficiency in T2D patients have defective control of intracellular bacteria such as *B. pseudomallei* due to reduced production of protective cytokines such as IL-12 and IFN- $\gamma^{521}$ . More recently, a higher frequency of *Mtb*-stimulated NK cells expressing TNF- $\alpha$  and IL-17 were found in TB patients with diabetes<sup>522</sup>. Different experimental approaches and the use of various models are likely to account for inconsistencies in findings between these studies. Broncho-alveolar-lavage fluid of patients with T2D contained significantly higher number of NKT cells compared to TB patients without T2D<sup>523</sup>.

Elevated levels of GM-CSF in diabetic patients have been attributed to the increased numbers of circulating DCs in diabetic patients<sup>506,524</sup>. However, dissemination of *Mtb* from lungs to lymph nodes as well as the emergence of *Mtb*-specific IFN- $\gamma$  producing T cells were delayed in diabetic mice<sup>525</sup>. Yamashiro, *et al.* <sup>505</sup> reported significantly reduced IFN- $\gamma$  production 14 days post infection with Mtb in T2D mice indicating impeded adaptive immune response. However, the reverse was true when human peripheral blood mononuclear cells (PBMC) from comorbid T2D patients were stimulated with *Mtb* antigens; ESAT-6 and CFP10, *in vitro*<sup>247</sup>. The authors reported exaggerated expansion of CD4<sup>+</sup> Th1 cells and higher production of Th1 plasma cytokines including IFN- $\gamma$  and TNF- $\alpha$  in T2D patients following antigen stimulation. A subsequent study by the same group<sup>526</sup> further extended the understanding of cytokine expression levels in TB patients with T2D. Higher Th1, Th2 as well as Th17 cytokine levels were associated with T2D. Interestingly, elevated cytokine levels were not influenced by age or sex of the subject but demonstrated a strong positive correlation with HbA<sub>1C</sub> levels. In contrast, significantly lower *ex-vivo* production of IFN-y found in active TB patients (both with and without diabetes) contradicts the previously seen elevated Th1 cytokine levels in TB/T2D patients<sup>527</sup>. Lower Th1:Th2 cytokine ratios in diabetic TB patients and this Th2 shift may explain why diabetic patients in general fail to control the disease<sup>510</sup>. Similarly, *in vitro* stimulation of human PBMC from TB patients in the presence of 40mmol/L glucose with either H37Rv lysate, heat-killed *Candida albicans* or LPS increased the expression of Th1 cytokines except IFN- $\gamma$ , IL-17A and IL-22<sup>507</sup>. In addition, elevated levels of CD8<sup>+</sup>T cells producing IFN- $\gamma$ , IL-2 & IL-17 have also been reported in pulmonary TB patients with T2D. Intriguingly, these CD8<sup>+</sup>T cells were associated with markedly reduced expression of cytotoxic makers such as perforin and granzyme B upon *Mtb*-derived antigen stimulation<sup>522</sup>, resulting in impaired cytotoxicity. In contrast to active TB, T2D individuals with LTBI have been characterised by lower Th1, Th2, Th17<sup>528</sup> and CD8<sup>+</sup>T cell counts<sup>529</sup>. Neutralization of either IL-10 or TGF- $\beta$  resulted in elevated Th1 and Th2 cell counts upon *Mtb* antigen stimulation<sup>528</sup>. A more recent study using integrative analysis of blood gene expression levels, plasma cytokine levels as well as clinical data revealed a distinct signature of plasma cytokine levels and an association with neutrophilic inflammation<sup>530</sup>.

However, the overall evidence suggests that the specificity and robustness of the immune response rather than the quantity of immune cells or cytokine levels are vital for a successful immune response against *Mtb* in comorbid T2D individuals. The conflicting findings among different studies make urgent the necessity to understand the complex underlying mechanisms that drive TB susceptibility in T2D.

# 1.3.3 Animal models of diabetes

Rationally designed new TB vaccine candidates are urgently required to meet the challenges of global TB eradication, especially in the context of TB/T2D comorbidity. Correlates of protection from human BCG vaccination are still incompletely understood and hence vaccine development is largely limited to correlates derived from animal studies. It is therefore crucial to include models of T2D into the global preclinical TB vaccine evaluation pipeline.

The majority of mouse models currently being used in TB research are based on autoimmune destruction of pancreatic beta cells thus resembling T1D. The STZ-induced model of T1D is vastly used as it offers a cheap and relatively cheap model of diabetes not only in small animals but also in NHPs<sup>531</sup>. In addition to the beta cell destruction, STZ also causes reduction in cellular ATP levels and subsequent inhibition of insulin production<sup>532</sup>. Despite its wide use in infectious disease research including in TB<sup>505,506</sup>, STZ can be toxic to organs including lungs thus affecting the organ microenvironments<sup>533</sup>. The most commonly used model of T1D is the non-obese diabetic (NOD) mice<sup>534</sup>. NOD mice develop diabetes as a result of insulitis characterised by CD4<sup>+</sup>, CD8<sup>+</sup> T cells, B cells and NK cell infiltration in pancreatic islet cells

followed by T cell mediated destruction of beta cells<sup>535</sup>. Compared to STZ- and other chemicalinduced diabetic models, NOD mice are expensive to maintain. In addition, a mutation in the *insulin 2* gene in the C57BL/6NSlc strain generates a genetically-induced insulin-dependent diabetes mouse model<sup>536</sup>.

Unlike type 1, T2D is characterised by insulin resistance,  $\beta$ -cell dysfunction and is strongly linked to obesity<sup>438</sup>. Obesity can be induced by genetic manipulation or high-fat diet feeding. Lep<sup>ob/ob</sup> and Lep<sup>db/db</sup> mice develop severe obesity due to a mutated leptin protein and leptin receptor, respectively<sup>537,538</sup>. Despite being hyperglycaemic and obese, due to the abnormalities in insulin secretion and lack of complete  $\beta$ -cell dysfunction, these models are not completely representative of human T2D. In addition to the above described monogenic obesity models, there are a number of polygenic models of obesity that are being currently used in animal research which include KK mice, OLETF rats, NZO mice, TallyHo/Jng mice and NoncNZO10/Ltj mice<sup>539</sup>. The first diet-induced model of T2D in C57BL/6 mice was described by Surwit and colleagues in 1988<sup>540</sup>. The 6 months feeding of high-fat induced obesity, hyperinsulinemia and glucose intolerance in mice. However, the fat content (35.8%) in the diet used by Surwit, et al, markedly exceeds the typical dietary intake in developed nations (60% energy vs 34% energy)<sup>541</sup>. In addition, it is now recognised that consumption of diets containing refined carbohydrates (energy-dense diet; EDD) significantly contributes to the development of T2D as a result of hyperinsulinemia-mediated pancreatic beta cell exhaustion followed by insulin deficiency<sup>542</sup>.

In this current study, we utilise a diet induced murine model of type 2 diabetes (DIMT2D) which encompasses a spectrum of cardinal metabolic and pathophysiological features of human T2D<sup>543</sup>. In this model, thirty weeks of EDD (23% fat, 19% protein, 50.5% dextrose and 7.5% fiber) intervention, leads to the development of increased fasting blood glucose levels, insulin resistance (as characterised by increased pancreatic islet area and elevated pancreatic insulin levels suggestive of hyperinsulinemic state) and higher HbA1c levels<sup>543</sup>. In addition, EDD-fed mice present with increased adiposity, circulating cholesterol and triglycerides and hepatic steatosis. Also, substantially high pro-inflammatory MCP-1 and IL-6 levels are observed in EDD-fed animals<sup>543</sup>. Collectively, these findings provide evidence that this DIMT2D closely resembles human T2D and incorporates metabolic dysregulation and systemic inflammation seen in the clinical disease, thus providing a useful and clinically relevant model to study comorbid infectious diseases such as TB in the context of T2D.

# 1.4 Project aims

The rapid emergence of drug-resistant *Mtb* strains and comorbid immunosuppressive conditions such as HIV/AIDS and diabetes; the two most potent risk factors predisposing *Mtb* infection and disease reactivation, provide a great challenge to the global control of TB. Although, the depletion of CD4<sup>+</sup> T cells, which is a main feature of HIV infection, is an important contributor to the increased risk of reactivation of latent TB, a significant knowledge gap in understanding the intricate mechanisms driving TB susceptibility in both HIV/AIDS and diabetics, remains.

Due to the incomplete protection against pulmonary TB, BCG; the only approved TB vaccine to date, is not the ideal vaccine for the global control of TB. A next generation anti-tuberculosis vaccine candidate should induce long-lasting immunity or even achieve a complete sterile eradication while being safe in immunocompromised individuals.

In this project, I aimed to investigate the underlying mechanisms of increased susceptibility to TB and vaccine mediated protection against TB in HIV/AIDS and T2D. To this end, I used an ear dermis infection model of LTBI reactivation and a diet induced murine type 2 diabetes model. My specific aims and objectives were:

**1**. To evaluate whether novel recombinant BCG vaccines could prevent the reactivation of latent lymphatic murine tuberculosis

**2**. To decipher the mechanisms driving increased TB susceptibility and to evaluate the safety and protective efficacy of experimental anti-TB vaccines expressing immunodominant *Mtb* antigens in the context TB/T2D

**3**. To investigate if protective anti-*Mtb* immunity in T2D mice can be restored by using the anti-diabetic drug metformin as an adjunctive TB therapy

# Chapter 2

# **Materials and Methods**

# 2.1 Bacteria

#### 2.1.1 Bacterial strains

All mycobacterial strains used in this study are listed in Table 2-1.

Bacteria/ strain	Characteristics	Resistance	Source
M. bovis			
			Mario AF Valdez (CIATEJ)
BCG Pasteur	wild-type (wil) strain of BCG		Nick West (UQ)
BCG Δ1419C	BCG Pasteur devoid of the cyclic di-GMP phosphodiesterase	Hygromycin	Mario AF Valdez (CIATEJ)
BCG::GFP	WT BCG expressing green fluorescent protein	Kanamycin	Camille Locht (Institut Pasteur de Lille)
BCG::RD1	BCG containing RD1 from Mtb	Hygromycin	Roland Brosch (Institut Pasteur)
BCG::RD1 ESAT-6 Δ92-95	BCG expressing C-terminal truncated (4 amino acids) ESAT6 protein from <i>Mtb</i> RD1 region	Hygromycin	Roland Brosch (Institut Pasteur)
BCG::OVA- mCherry	BCG expressing OVA protein tagged with fluorescent mCherry	Hygromycin/ Kanamycin	Jamie Triccas (USyd)
Mtb			
H37Rv	WT strain of <i>Mtb</i>	Ampicillin	BEI Resources

Table 2-1: Bacterial strains used in this study

Abbreviations: CIATEJ, Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisc; UQ, University of Queensland; USyd, University of Sydney; BEI Resources, Biodefense and Emerging Infections Research Resources Repository.

#### 2.1.2 Bacterial growth conditions

*Mycobacterium tuberculosis* strain H37Rv (ATCC no. 27294) and all BCG strains were grown as previously described<sup>544</sup>. Briefly, 500 µl to 1 ml of thawed strain stock was transferred to a 50 ml falcon tube containing 10 ml of Middlebook 7H9 broth (Becton Dickinson) supplemented with 0.2% w/v glycerol, 0.05% w/v, Tween 80, 10% v/v, Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton Dickinson) and appropriate antibiotics (**Table 2-1**). The tube was incubated in a shaking platform at 37°C with mild agitation (100-125 rpm) until a uniform turbidity is developed (indication of bacterial growth). The culture was then expanded by transferring into a sterile, disposable plain bottom 250 ml Erlenmeyer cell culture flask (Corning) containing ~100 ml of 7H9 broth. The flasks were further incubated under mild agitation for 3-5 days. Midlogarithmic (OD<sub>600nm</sub> 0.6-0.9) bacterial cultures were harvested by centrifugation for 12 mins at 3500 rpm (Allegra X-30, Rotor SX4400, Beckman Coulter). Cell pellets were washed twice with sterile PBS and re-suspended in 50 ml of 15% (v/v) glycerol and stored in 1 ml cryovials at -80 °C. For colony forming unit (CFU) enumeration, serial dilutions were performed and 100 µl from each concentration was

plated on Middlebrook 7H11 agar (Becton Dickinson) supplemented with 10% v/v Middlebrook OADC enrichment (Becton Dickinson) and 0.2% v/v glycerol. If required, the agar was supplemented with antibiotics listed in **Table 2-1**. Plates were incubated at 37 °C for 3-4 weeks before counting.

# 2.2 Experimental animals

# 2.2.1 Mice – husbandry and housing

All animal experiments were approved by the Animal Ethics Committee (AEC) of James Cook University (Project approval numbers: A2400 & A2403) and conducted according to the National Health and Medical Research Council (NHMRC) Australian code for the care and use of animals for scientific purposes (2013). Male C57BL/6 mice were obtained from in-house breeding at the Australian Institute of Tropical Health and Medicine (AITHM) animal facility or were purchased from the Animal Resource Centre (ARC; Perth, WA). All mice were housed under specific pathogen free (SPF) conditions in individually ventilated cages (18-22 °C, relative humidity of 70-75%, 12-hour light/ dark cycle) in the AITHM animal facilities. Animals that were imported from other animal facilities were kept in a quarantine room at the Immunogenetic Research Facility (IGRF) at JCU and acclimatised for at least 7 days before being used for experiments. All vaccination procedures with were performed inside a class II biological safety cabinet in the AITHM PC2 (physical containment level 2, also known as biological safety level 2; BSL2) animal facility. Mice to be infected with Mtb were moved to the AITHM PC3 facility and kept in IsoCage N – Biocontainment System (Techniplast, Italy). Mice were euthanised by CO<sub>2</sub> asphyxiation or cervical dislocation. Biosafety approvals were granted for all experiments by the institutional biosafety committee of JCU (JCUIBC-170210-006 & JCUIBC-160922-014).

Mouse strain	Description	Source
C57BL/6	WT C57BL/6 mice	JCU & ARC
C57BL/6 OT-I	C57BL/6 transgenic mice with CD8 <sup>+</sup> T cells recognising ovalbumin peptide residues 257-264 (OVA <sub>257-264</sub> ) in the context of H2K <sup>b</sup> (MHC class I)	UQ
C57BL/6 OT-II	C57BL/6 transgenic mice with CD4 <sup>+</sup> T cells recognising OVA peptide residues 323-339 (OVA <sub>323-339</sub> ) in the context of I-A <sup>b</sup> (MHC class II)	JCU
C57BL/6 CD45.1+ OT-I	OT-I mice carrying the congenic CD45.1 leukocyte marker	JCU
C57BL/6 CD45.1 <sup>+</sup> OT-II	OT-II mice carrying the congenic CD45.1 leukocyte marker	JCU

 Table 2-2: Mouse strains used in this study

#### 2.2.2 Induction and confirmation of type 2 diabetes

This murine T2D model was developed and extensively characterized using male C57BL/6 mice<sup>543</sup>. Moreover, estrogen protects female mice from developing high-fat diet induced adipocyte hypertrophy, liver steatosis and from becoming insulin resistant<sup>545</sup> and thus female mice were not included. At 4-6 weeks of age, male C57BL/6 mice were randomly separated into two dietary groups and baseline bodyweights were recorded. One group of mice was fed with *ad libitum* amount of energy-dense diet (EDD, SF03-030, Speciality Feed, Western Australia) made of 23% fat, 19.4% protein, 48% refined carbohydrate and 9.2% fibre. The other group received a calorie-restricted standard rodent diet (SD, 3.5g or 13.65 kcal per mouse per day) with 13.6% protein, 4% fat, 64.3% carbohydrate and 9.4% fibre (SF08-020, Speciality Feed, Western Australia). All feed was irradiated at 25kGy and supplemented with increased vitamin premix (×1.5) to compensate the loss of nutrients during the irradiation process. Following 30 weeks of diet intervention, mice from both groups were assessed for body weight gain, fasting blood glucose levels and glucose tolerance to determine the diabetic status.

To measure fasting blood glucose levels, mice were fasted for 6 hours prior to the test. The tip of the tail (<1mm) was cut using a sterile scalpel blade and a drop of blood (~5  $\mu$ l) was massaged out and used on an Accu-Check Performa blood glucose monitor (Roche, Australia). Similarly, for glucose tolerance test (GTT), the baseline blood glucose levels were measured on 6-hours fasted mice. Hundred  $\mu$ l of 2 g/Kg glucose solution was administered i.p. using a 27G needle and blood glucose levels were measured at 15, 30, 60 and 120-mins post administration intervals, as described above. The hyperglycaemic threshold was calculated based on the area under the curve (AUC) from GTT readings. Briefly, any EDD-fed mice with an AUC level higher than the upper 99% confidence interval for the mean of the age-matched SD-fed control group, was considered diabetic<sup>543,546</sup>.

#### 2.2.3 Immunization and infection

Prior to vaccination, thawed BCG vaccine strain stocks were sonicated (for 30 sec) and vortexed to disrupt any bacterial clumps and resuspended in PBS to achieve the appropriate CFU/ml required for immunisation. C57BL/6 mice were immunised with  $5x10^5$  CFU via intranasal (i.n.), subcutaneous (s.c.), and intramuscular (i.m.) routes in 30, 100 and 50 µl volumes, respectively, except in Chapter 3 where  $1x10^6$  CFU was used for BCG s.c. delivery into the tail base of the animals. For i.t. BCG vaccination, mice were anesthetized via i.p. injections of Xylazine (5 mg/kg) and Ketamine (50 mg/kg) or using isoflurane (induction- 5%).

and maintenance- 3%), the tongue was drawn out of the oral cavity with blunted forceps and 50  $\mu$ l of inoculum was administered into the oropharynx with the nostrils covered until the inoculum was inhaled. To determine protective efficacy, 60 days post-vaccination (p.v.) mice were challenged via the aerosol route with a very low dose of *Mtb* H37Rv (10-20 CFU per mouse) using a Glas-Col inhalation exposure system as described by Ordway, *et al.* <sup>547</sup>. Bacterial titration was performed to determine the required volume of mouse-adapted *Mtb* stock to achieve a very-low dose infection of 10-20 CFU per mouse. To determine the actual infectious dose, 4-5 mice were sacrificed at one day post-infection (p.i.) and lung organ homogenates were plated on 7H11 agar plates supplemented with OADC and appropriate antibiotics listed in **Table 2-1**. For intradermal (i.d.) infection in Chapter 3, mice were anesthetized using Xylazine and Ketamine and 1×10<sup>2</sup> CFU *Mtb* H37Rv were subsequently injected in to the ear dermis intradermally (i.d.) in a volume of 50  $\mu$ l<sup>425</sup>.

#### 2.2.4 Metformin and isoniazid administration

Drugs were administered at 500 mg/kg metformin (MET, Sigma) and 10 mg/kg isoniazid (INH, Sigma) alone or in combination (MET+INH) via the drinking water. Treatment commenced seven days post aerosol *Mtb* challenge. 1.25 mg/ml MET in drinking water delivers a dose of approximately 250 mg/kg to mice<sup>548</sup>. The concentration of each drug in drinking water to deliver the said doses was calculated accordingly (i.e., 2.5 mg/ml MET -> 500 mg/kg). Water bottles containing drugs were changed every 4-6 days.

# 2.2.5 In vivo CD4<sup>+</sup> T cell depletion

CD4<sup>+</sup> T cells were depleted from C57BL/6 mice by weekly injections of 200  $\mu$ g of purified anti-CD4 mAbs (BioXCell; GK 1.5) i.p. in 200  $\mu$ l of sterile PBS. The first dose of anti-CD4 mAbs was given immediately after *Mtb* infection.

#### 2.3 Histology

Perfused or un-perfused lungs from naïve, treated, vaccinated or *Mtb*-infected animals were fixed for 24 h in 10% neutral buffered formalin and then dehydrated in 70% ethanol and embedded in paraffin for histological analysis. Four  $\mu$ m sections were cut, deparaffinised and stained with hematoxylin and eosin (H&E) and/ or acid-fast Ziehl-Neelsen (ZN). Stained lung sections were visualised using either a light microscope (BX43F; Olympus) or a research stereo microscope system (SZX2-ILLT; Olympus) and cellSens Imaging Software (Olympus). Images were captured at 10×, 25× or 1000× (oil immersion) and used to measure the total lung area, for representative images and to detect *Mtb*, respectively. Tissue infiltration/ inflammation was measured by calculating the percentage of cell dense area using ImageJ software. Briefly, the total lung area (in  $10 \times$  figures) was first measured using the 'Freehand selections tool'. Cell dense area (dark blue; in  $10 \times$  figures) was then highlighted by adjusting the Hue, Saturation and Brightness parameters in 'Threshold Colour' function in the ImageJ software.

% of tissue infiltration 
$$= \frac{Cell \, dense \, area}{Total \, lung \, area} \times 100$$

# 2.4 Determination of organ bacterial loads

Aseptically removed lung tissues were homogenised in sterile sample bags containing 1 ml of sterile PBS/ 0.05% Tween 80. Serial dilutions of tissue homogenates were plated on 10% OADC enriched 7H11 agar plates supplemented with 10  $\mu$ g/ul cycloheximide or appropriate antibiotics (50  $\mu$ g/ml Hygromycin and 20  $\mu$ g/ml Ampicillin for rBCG strains and *Mtb*, respectively). Thiophene-2-carboxylic acid hydrazide (TCH, 2  $\mu$ g/ml; Sigma) was added to 7H11 agar plates to restrict the growth of BCG strains for *Mtb* culture. Agar plates were sealed with parafilm, wrapped in aluminium foil and incubated aerobically for 3-4 weeks at 37°C. Colonies were counted and the total CFU per organ was calculated based on dilution factors and organ size.

# 2.5 Determination of blood cytokine levels

Blood was collected via cardiac puncture into Z-gel tubes (Sarstedt). Serum was prepared by centrifugation of clotted blood at 10,000 *g* for 5 mins (Microfuge 16, Rotor FX241.5P or Microfuge 20R, FA241.5P both from Beckman Coulter) and stored at -20°C. Serum samples from *Mtb* infected animals were filtered using 0.2 µm SpinX columns (Sigma) and stored at -20°C for further downstream analyses. Serum cytokine levels were determined using Bio-Plex Pro<sup>TM</sup> Mouse Cytokine 23-plex assay (BioRad) according to the manufacturer specifications and analysed using a calibrated MagPix (Luminex) instrument.

#### 2.6 Flow cytometry

#### 2.6.1 Single cell preparation

#### 2.6.1.1 Lung

Lungs were perfused by flushing 10-20 ml of sterile PBS through the heart right ventricle of the heart. The tissues were excised, mechanically disrupted and digested for 30 mins at 37°C with sterile RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum

(FBS) (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), 7.5 µg/ml Collagenase D (Sigma), 1.75 µg/ml Collagenase VIII (Sigma) and 200 µg/ml DNaseI (Sigma). Digested tissues were gently passed through a 70 µm cell strainer using 3cc syringe plunger to obtain single cells. Cells were washed and red blood cells (RBCs) were lysed with 3 ml of homemade ACK buffer (1L of 10X buffer contains 82.6 g NH<sub>4</sub>Cl, 10 g KHCO<sub>3</sub> or 8.4 g NaHCO<sub>3</sub> and 372 mg Na<sub>2</sub>EDTA) or BD FACS Lysing Solution (BD Biosciences) for 2 mins at room temperature. Lysing buffer was neutralized with RPMI 1640 containing 5-10% FBS. Cell were washed with ice-cold RPMI 1640 or FACS buffer (PBS/ 5% FBS and 0.1% NaN<sub>3</sub>), pelleted and resuspended in the appropriate buffer.

# 2.6.1.2 Intra-airway luminal cells

Bronchial lavage (BAL) was used to recover intra-airway luminal cells for downstream flow cytometric analysis. After exposing trachea, a small incision was made at the proximal end of the trachea and 1 ml of ice-cold PBS was injected and extracted carefully using a 18G blunt needle. The process was repeated for 3 times to collect a total of 3 ml of BAL fluid (BALF). The cell suspension was centrifuged at 1500 rpm (Allegra X-30, Rotor SX4400, Beckman Coulter) for 5 mins at 4 °C, pelleted and used for downstream FACS staining.

#### 2.6.1.3 Lymph nodes and spleens

Superficial cervical, mediastinal and inguinal lymph nodes (LNs) were removed and collected in cold RPMI 1640. To prepare single cells, LNs were gently teased through a 70  $\mu$ m cell strainer. For spleens, RBCs were lysed using either ACK buffer or BD FACS Lysing Solution for 3-5 mins at RT. Cells were spun at 1500 rpm (Allegra X-30, Rotor SX4400, Beckman Coulter) for 5 mins at 4 °C and resuspended in cold FACS buffer for downstream staining.

#### 2.6.2 Surface staining

Antibody staining was carried out in 96-well U bottom microwell plates (Sarstedt). Single cells were resuspended in 100  $\mu$ l of PBS containing Horizon<sup>TM</sup> Fixable Viability Stain 780 (BD Biosciences) and incubated for 10-15 mins at RT in dark. This step facilitates the exclusion of dead cells. Cells were washed twice at 1500 rpm (all washing steps during FACS staining were carried out using Allegra X-30, Rotor S6056, Beckman Coulter) with cold FACS buffer and stained with surface antibodies and Fc-block<sup>TM</sup> (purified rat anti-mouse CD16/CD32, BD Biosciences). Staining was performed in 50  $\mu$ l FACS buffer for 30 mins on ice or at 4 °C using the fluorochrome-conjugated primary antibodies or tetramers listed on **Table 2-3**. To stain with *Mtb*-specific tetramers, cells were first incubated with ESAT-6 or Ag85.B-tetramers in 30  $\mu$ l

at RT for 60 mins, followed by an incubation with TB10.4- tetramer and CXCR3 in 30  $\mu$ l on ice or at 4 °C for further 15 mins. The rest of the surface antibodies in 30  $\mu$ l FACS buffer was added and incubated for another 20 mins on ice or at 4 °C. For MAIT cells, 6FP and 6-OP-RUtetramers, TCR $\beta$  and Fc-block<sup>TM</sup> in 50  $\mu$ l FACS buffer was added and incubated at RT for 30 mins. The rest of the surface antibodies was added in 50  $\mu$ l FACS buffer without washing the cells and incubated on ice or at 4 °C for further 30 mins. After incubation, cells were diluted in FACS buffer and subsequently washed twice. At this stage, cells can be fixed with 100  $\mu$ l fixation buffer (4% PFA, eBioscience) for 10-15 mins at RT and proceed for intracellular staining (ICS) or resuspended in 150  $\mu$ l of FACS buffer containing 5  $\mu$ l of CountBright Absolute Counting Beads (Invitrogen). Compensation for multicolor assay was performed using either single colour stained samples or BD CompBeads Set (BD Bioscience). Samples were acquired using a BD CantoII flow cytometer or a BD LSRFortessa X-20 flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software version 10 (Treestar, CA).

#### 2.6.3 Intracellular staining (ICS)

The Foxp3/ Transcription Factor Staining Buffer Set (eBioscience) was used for ICS according to the manufacturer's instructions. In brief, after surface staining, cells were fixed with fixation buffer and washed twice with permeabilization buffer (eBioscience; 0.1% saponin, 0.09% sodium azide). Cells were incubated with 200  $\mu$ l of permeabilization buffer for 20 mins at RT and subsequently spun down and stained with antibodies against intracellular cytokines/ transcription factors (**Table 2-3**) in 50  $\mu$ l permeabilization buffer. Cells were incubated on ice or at 4 °C for 30 mins before washing twice with permeabilization buffer. Cells were then fixed, resuspended in 150  $\mu$ l of FACS buffer containing 5  $\mu$ l of counting beads, acquired and analysed as described above.

Antibody	Clone	Conjugate	Dilution/ concentration	Source
Surface markers				
CD3e	500A2	AF700	1:100/ 1:200	BD Phamingen
CD4	GK1.5	BUV395	1:200/ 1:400	BD Phamingen
	RM4-5	BUV737	1:200/ 1:400	BD Phamingen
	RM4-4	PE	1:200/ 1:400	StemCell Tech.
CD8	53-6.7	BV510	1:200/ 1:400	BD Phamingen
CD11b	MI/70	PE-Cy7	1:200	BD Phamingen

Table	2-3:	List o	of ant	ibodies
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	MI/70	AF647	1:200	BD Phamingen
CD11c	HL3	BUV395	1:200	BD Phamingen
	N418	PerCP-Cy5.5	1:200	BD Phamingen
CD14	Sa2-8	PE	1:200	eBioscience
CD19	1D3	PE-CF594	1:200/ 1:400	BD Phamingen
CD40	3/23	BV421	1:200	BD Phamingen
CD44	IM7	BV421	1:200/ 1:400	BD Phamingen
	IM7	PE-Cy5	1:200/ 1:400	BD Phamingen
CD45.1	A20	BV605	1:200/ 1:400	BD Phamingen
CD45.2	104	BV650	1:200/ 1:400	BD Phamingen
CD62L	MEL-14	PE-Cy7	1:200/ 1:400	BD Phamingen
CD64a/b	X54-9/7.1	BV786	1:50	BD Phamingen
CD69	H1.2F3	FITC	1:200	BD Phamingen
	H1.2F3	PE-CF594	1:200	BD Phamingen
CD80	16-10A1	BV786	1:200	BD Phamingen
CD86	GL-1	PerCP-Cy5.5	1:200	BD Phamingen
CD103	M290	APC	1:200/ 1:400	BD Phamingen
	M290	BV605	1:200/ 1:400	BD Phamingen
CD107a	1D4B	BV711	1:200	BD Phamingen
	eBio1D4B	PerCP-eF710	1:200	BD Phamingen
CD183 (CXCR3)	CXCR3-173	Bv786	1:100/ 1:200	BD Phamingen
CD209a (DC-SIGN)	5H10	BV711	1:200	BD Phamingen
CD282 (TLR2)	6C2	AF647	1:200	BD Phamingen
CD284 (TLR4)	MTS510	PE	1:200	BD Phamingen
CD335 (NKp46)	29A1.4	BV711	1:200/ 1:400	BD Phamingen
	29A1.4	BV650	1:200/ 1:400	BD Phamingen
TCRβ	Н57-597	PE	1:200/ 1:400	BD Phamingen
	Н57-597	FITC	1:200/ 1:400	eBioscience
ΤCRγδ	GL3	PE	1:200	BD Phamingen
TCRVa2	B20.1	APC	1:100/ 1:200	eBioscience
E-Cadherin	36/E	BV421	1:100	BD Phamingen
F4/80	T45-2342	BV711	1:200	BD Phamingen
	BM8	FITC	1:200	BD Phamingen
	BM8	PerCP-Cy5.5	1:200	BD Phamingen
KLRG1	2F1	AF488	1:200	BD Phamingen
LFA-1	H155-78	PerCP-Cy5.5	1:200	BD Phamingen
Ly-6G	1A8	PerCP-Cy5.5	1:200	BD Phamingen
MARCO	ED31	FITC	1:10	BioRad

MerTK	DS5MMER	SuperBright 436	1:100	eBioscience
I-A/I-E (MHCII)	M5/114.15.2	BV605	1:200	BD Phamingen
Siglec-F	E50-2440	PE	1:200	BD Phamingen
Intracellular markers	1			
CD206 (MMR)	CO68C2	AF647	1:100	Biolegend
GranZB	NGZB	FITC	1:100	eBioscience
IFNγ	XMG1.2	BV786	1:100	BD Phamingen
IL-17A	TC11-18H10	PE-CF594	1:100	BD Phamingen
IL-22	Poly5164	APC	1:100	Biolegend
iNOS	CXNFT	APC	1:100	eBioscience
Ki67	SolA15	PE-eFlour 610	1:100	eBioscience
T-bet	O4-46	PerCP-Cy5.5	1:100	BD Phamingen
Tetramers	1			
mMR1 5-OP-RU		APC	1:100	NIH
mMR1 6-FP		APC	1:100	NIH
H-2K(b) Mtb TB10.44-11		FITC	1:50	NIH
I-A(b) Mtb Ag85B280-294		PE	1:50	NIH
I-A(b) Mtb ESAT64-17		PE	1:50	NIH
I-A(b) human CLIP		PE	1:100	NIH
Functional grade purified antibodies				
CD3e (In vitro T cell	145-2C11		4 µg/ml	eBioscience
stimulation)	145-2C11		4 µg/ml	BD Phamingen
CD4 ( <i>In vivo</i> CD4 <sup>+</sup> T cell depletion)	GK1.5		200 µg per mouse per week	BioXCell

# 2.7 In vitro lung cell stimulation and infection

96-well U bottom microwell plates (Sarstedt) were coated with 200 µl of 4 µg/ml anti-CD3e mAb (145-2C11) in PBS overnight at 4 °C. The next day, plates were washed 3 times with sterile PBS before adding cells. RBC depleted lung single cells ( $1-5 \times 10^6$  cells) were resuspended in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol (Gibco) and 3 µg/ml Brefeldin A (eBioscience) and were added to pre-coated 96-well U bottom microwell plate. Cells were cultured for 5-6 hours at 37 °C in 5 % CO<sub>2</sub>. Some cells were also infected with *Mtb* H37Rv or BCG strains for 4 hours at a multiplicity of infection (MOI) of 1 to detect cytokines and other intracellular markers. After stimulation, cells were washed twice with cold FACS buffer before being stained for both surface and intracellular markers. In addition, isolated bone marrow derived macrophages (BMDMs) and dendritic cells (BMDCs) were

infected with BCG and *Mtb* strains at a MOI of 1 for 4 to 24 hours and stained for variety of innate surface markers.

#### 2.8 Generation of bone marrow derived macrophages and dendritic cells

Bone marrow derived cells were prepared from mice as described previously<sup>549</sup>. Briefly, femur and tibia were excised, excess muscle tissues were removed and cleaned with 70% ethanol for 2-5 mins. Bones were washed with sterile PBS before transferring to ice-cold RPMI 1640. The tips of the bones were cut and marrows were flushed with RPMI 1640 supplemented with 10% FBS using a 27G needle. Cells were gently passed through a 70 µm cell strainer and resuspended in complete medium.

For macrophage differentiation,  $\sim 2 \times 10^6$  cells in 10 ml of complete macrophage medium (DMEM supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1mM sodium pyruvate, 50 µM 2-mercaptoethanol and 40 ng/ml recombinant mouse macrophage colony stimulating factor (rM-CSF; Invitrogen) were seeded per petri dish. Cells were cultured at 37 °C in 5% CO<sub>2</sub> for 7 days with regular medium changes. Adherent cells were removed using 5 ml of TrypLE express (Gibco), cell purity was assessed using CD11b and F4/80 mAbs by flow cytometry.

Similarly, to generate DCs,  $\sim 2 \times 10^6$  bone marrow cells were seeded per petri dish in 10 ml of complete DC medium containing RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1mM sodium pyruvate, 50 µM 2-mercaptoethanol and 20 ng/ml recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; Invitrogen). After regular medium changes, non-adherent cells were harvested on day 8 and assessed for cell purity using CD11b, CD11c and MHC II mAbs.

# 2.9 LPS instillation

Anesthetized mice received 5  $\mu$ g of LPS (Sigma) in 50  $\mu$ l sterile PBS intratracheally, as described above.

# 2.10 Cell enrichment

# 2.10.1 CD11b<sup>+</sup> macrophages

BMDMs were enriched using EasySep<sup>TM</sup> Mouse CD11b Positive Selection Kit II (StemCell Technologies) according to manufacturer's instructions. In brief, cell counts were adjusted to  $1 \times 10^8$  cells per ml and treated with 50 µl/ml of sample. The selection cocktail was prepared by

mixing equal volumes of component A and B and 50  $\mu$ l of the cocktail per 1 ml sample was added and incubated at RT for 5 mins. Subsequently, RapiSpheres particles were added to the sample and place the sample tube into the EasySep magnet. After 3 mins of incubation at RT, the supernatant was poured off by inverting the magnet and the tube in one continuous motion. The tube contained the enriched CD11b<sup>+</sup> cells. Cells were resuspended in desired medium.

# 2.10.2 CD11c<sup>+</sup> dendritic cells

CD11c<sup>+</sup> cells including DCs were enriched using EasySep<sup>TM</sup> Mouse CD11c Positive Selection Kit II (StemCell Technologies) as described above.

# 2.10.3 CD4<sup>+</sup> and CD8<sup>+</sup> T cells

CD4<sup>+</sup> and CD8<sup>+</sup>T cells were isolated from single cell suspensions of spleens and LNs using EasySep<sup>TM</sup> Mouse CD4<sup>+</sup> T Cell Isolation Kit and EasySep<sup>TM</sup> Mouse CD8<sup>+</sup> T Cell Isolation Kit (StemCell Technologies), respectively. Unlike CD11b and CD11c positive selection kits, CD4<sup>+</sup> and CD8<sup>+</sup> T cell isolation kits employed a negative selection method, thus resulting in unlabelled/ untouched CD4<sup>+</sup> and CD8<sup>+</sup> T cells for downstream applications. CD3, CD4 and CD8 surface markers were used to determine the purity of isolated cells by flow cytometry.

# 2.11 T cell proliferation assay

CD11c<sup>+</sup> BMDCs were infected with BCG vaccine strains for 2 hours at a MOI of 10 in RPMI 10% FBS. Cells were treated with 200  $\mu$ g/ml amikacin for further 2 hours to kill extracellular bacteria. After 16 hours, cells were harvested and incubated with 10  $\mu$ g/ml OVA<sub>257-264</sub> (SIINFEKL) and OVA<sub>323-339</sub> (ISQAVHAAHAEINEAGR) peptides for an hour at 37 °C. OVA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells were purified from C57BL/6 OT-I/II mice using EasySep<sup>TM</sup> Mouse CD4<sup>+</sup> and CD8<sup>+</sup> T Cell Isolation Kits (StemCell Technologies) and stained with 2  $\mu$ M VPD450 (BD Bioscience) or 5  $\mu$ M CTV (Invitrogen) following manufacturers' guidelines. Peptide-pulsed DCs were then co-cultured with VPD- or CTV-stained T cells at a ratio of 1:1. Following 72-hour incubation cells were stained for T cell lineage markers and acquired using flow cytometry. T cell division was analysed using the VPD450/ CTV fluorescent peaks in the 450/40 nm channel.

#### 2.12 Determination of cellular nitrite production

Nitrite levels in cell supernatants were measured using a commercially available Griess Reagent Kit (Invitrogen). BMDMs were seeded in 96-well U bottom microwell plates  $(1 \times 10^5$ 

cells per well in complete RPMI 1640 medium). BCG and *Mtb* strains were added to each well as per different MOIs for 24, 48 and 72 hours. Single lung cell suspensions from both unvaccinated and vaccinated mice were also seeded in 96-well U bottom microwell plates and infected with *Mtb* at a MOI of 1 for 24 hours. At the end of the designated time point, plates were centrifuged, supernatants were gently aspirated and filtered using 0.2 µm SpinX columns (Sigma). The Griess assay was performed as per the kit instructions. Reagents were prepared freshly and the absorbance of the nitrite containing samples was measured at 548 nm relative to the reference (background) sample using a spectrophotometer (FLUOstar Omega and SPECTROstar<sup>Nano</sup> from BMG LABTECH). The absolute nitrite concentrations were calculated using a standard curve.

# 2.13 In vitro macrophage phagocytosis assay

Phagocytic capacity of BMDMs were assessed by infecting  $1 \times 10^5$  BMDMs with BCG::GFP or incubating with Fluoresbrite<sup>TM</sup> 2 µm Yellow Green Microspheres (Polysciences) at MOIs of 1 and 10 for 4 hours at 37 °C in 5% CO<sub>2</sub>. After incubation cells were prepared for FACS staining. Live macrophages were gated using CD11b and F4/80. Cells positive for BCG::GFP or beads were detected in the FITC channel by flow cytometry.

#### 2.14 DNA and RNA extraction

Fresh or RNA*later* (Invitrogen)- preserved lung tissue sections and stool samples were used for both DNA and RNA extractions. The PureLink<sup>TM</sup> Microbiome DNA Purification Kit (Invitrogen) kit was used for microbial DNA extraction. For stool samples, 2-3 stool pellets (50-100 mg) were incubated with lysis enhancer and mechanically disrupted in sterile gentleMACS M tubes using a GentleMACS tissue dissociator (Miltenyi Biotec). Manufacturer instructions were followed for subsequent steps. A soil DNA extraction protocol was used to purify DNA from lung tissue sections. Initial mechanical disruption was performed using a GentleMACS tissue dissociator as described above. Purified DNA was stored at -20°C in Nuclease-Free Eppendorf tubes. Total RNA from lung tissue sections were extracted using the PureLink<sup>TM</sup> RNA Mini Kit (Invitrogen) and TRIzol<sup>TM</sup> reagent (Invitrogen). 1 ml of TRIzol reagent per 50-100 mg of tissue samples was used to homogenize the tissue samples in M tubes and a GentleMACS tissue dissociator. Purified RNA was stored in RNase-Free microfuge tubes. Purified DNA and RNA was quantified with the Qubit 4.0 Fluorometer (Invitrogen) using the Qubit dsDNA BR Assay Kit and the Qubit RNA HS Assay Kit (Invitrogen), respectively. DNA and RNA concentrations were subsequently adjusted using RNase-Free water for downstream assays.

# 2.15 16S rRNA-based microbiome analysis

DNA concentration was adjusted to 5 ng/µl. The 16S rRNA gene encompassing the V5 to V8 regions was targeted using the 803F (5'- TTAGAKACCCBNGTAGTC -3') and 1392wR (5'- ACGGGCGGTGWGTRC -3') primers<sup>550</sup> modified to contain Illumina specific adapter sequence (803F: 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGTTAGAKAC CCBNGTAGTC -3' and 1392wR: 5'-GTCTCGTGGGCTCGGGGTCTCGTGGGCT CGGAGATGTGTATAAGAGACAGACAGACGGGCGGTGWGTRC - 3'). The prokaryote primer pairs Prok\_SSU\_803F-1392wR amplifies the small subunit (SSU) ribosomal RNA of bacteria and archaea (16S), specifically the V5, V6, V7 and V8 regions. In *Escherichia coli*, it amplifies the 803-1392 region of the 16S gene.

Preparation of the 16S library was performed as described, using the workflow outlined by Illumina (#15044223 Rev.B). In the 1st stage, Polymerase Chain Reaction (PCR) products of ~466bp were amplified according to the specified workflow with an alteration in polymerase used to substitute NEBNext® UltraTM II Q5® Mastermix (New England Biolabs) in standard PCR conditions. Resulting PCR amplicons were purified using Agencourt AMPure XP beads (Beckman Coulter). Purified DNA was indexed with unique 8bp barcodes using the Illumina Nextera XT 384 sample Index Kit A-D (Illumina FC-131-1002) in standard PCR conditions with NEBNext® UltraTM II Q5® Mastermix. Indexed amplicons were pooled together in equimolar concentrations and sequenced on the MiSeq Sequencing System (Illumina) using paired end sequencing with V3 300bp chemistry in the Australian Centre for Ecogenomics (ACE) according to the manufacturer's protocol. Briefly, 2×300bp paired 16S reads were sequenced and run through FastQC for quality control, Trimmomatic<sup>551</sup> for adapter trimming and low quality base removal, QIIME<sup>552</sup> for operational taxonomy units (OTU) generation, and BLAST<sup>553</sup> for OTU identification. All OTUs from the provided biom file were aligned to the mouse reference genome mm10 using bowtie2554 with all aligned reads removed using SAMTools<sup>555</sup>. This process removed 77.47% of all sequence reads with only the non-aligning reads input into separate biom files for lung and stool samples. For each biom file, the taxonomic observation and metadata was added using the biom API<sup>556</sup> which was next loaded into the R package phyloseq<sup>557</sup>. Within phyloseq, the DESeq2<sup>558</sup> API was called and a list of most differentially expressed bacteria generated for all possible pairings of the four sets of conditions (naïve control, T2D, TB and T2D/TB). All subsequent plots were generated using ggplot2.

# 2.16 NanoString assay

The RNA integrity number (RIN) of purified RNA was assessed using the Bioanalyser 2100 (Agilent). A NanoString nCounter assay (NanoString Technologies) was performed using the mouse immunology panel v1 which contains total of 561 general immunology genes and 15 internal reference genes. This panel is considered to be ideal for infectious disease immune responses, autoimmune and allergy. This includes major class of cytokines and their receptors, chemokine ligands and receptors, IFNs and their receptors, TNF-receptor superfamily and Killer Ig-like Receptor (KIR) family genes. Standard manufacturer protocol was followed. Briefly, ~50 ng of high-quality RNA (RNA integrity number; RIN>7) was hybridized to NanoString probes and RNA-probe complexes were processed using nCounter Prep Station. The nCounter Digital Analyzer was used to count individual fluorescent barcodes and to quantify target mRNA molecules. Initial NanoString data quality control and normalization were performed in nSolver software (NanoString Technologies). The R software package limma<sup>559</sup> was used for further differential gene expression and comparative analyses.

# 2.17 Statistical analysis

Statistical analysis was performed, and graphs were generated using GraphPad Prism version 8 (CA, USA). Two and multiple parametric group analyses were carried out using Student's *t*-test and one-way or two-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's multiple comparison test, respectively. Not-normally distributed data was analysed using Mann-Whitney and Kruskal-Wallis tests. P < 0.05 was considered significant, unless otherwise stated.

# **Chapter 3**

# BCG vaccination prevents reactivation of latent lymphatic murine tuberculosis independently of CD4<sup>+</sup> T cells

This chapter titled 'BCG Vaccination Prevents Reactivation of Latent Lymphatic Murine Tuberculosis Independently of CD4+ T cells' has been published in *Frontiers in Immunology* on 21<sup>st</sup> March 2019 (DOI: 10.3389/fimmu.2019.00532).

#### 3.1 Abstract

Tuberculosis (TB) is a major global public health problem causing significant mortality and morbidity. In addition to approximately 10.4 million cases of active TB annually, it is estimated that about two billion people are latently infected with Mycobacterium tuberculosis (Mtb), the causative agent of TB. Reactivation of latent Mtb infection is the leading cause of death in patients with immunodeficiency virus (HIV) infection. The low efficiency of the only licensed anti-TB vaccine, Bacille Calmette-Guérin (BCG) to reduce pulomonary TB in adults contributes to this problem. Here we investigated if vaccination with conventional BCG or the genetically modified experimental BCGABCG1419c strain can prevent reactivation of latent lymphatic TB in a mouse model of induced reactivation, following the depletion of CD4<sup>+</sup> T cells, as it occurs in HIV+ individuals. Vaccination with conventional BCG and BCG $\Delta$ BCG1419c prevented reactivation of *Mtb* from the infected lymph node and the systemic spread of Mtb to spleen and lung. Prevention of reactivation was independent of vaccination route and was accompanied by reduced levels of circulating inflammatory cytokines and the absence of lung pathology. Our results demonstrate that vaccine-induced CD4<sup>+</sup> T cells are not essential to prevent reactivation of latent lymphatic murine TB, and highlight the need to better understand how non-CD4<sup>+</sup> immune cell populations participate in protective immune responses to control latent TB.

# 3.2 Background

Tuberculosis (TB) affects approximately 10.4 million people annually and is associated with 1.7 million deaths per year<sup>560</sup>. Despite the availability of effective anti-TB drugs, poor adherence to long treatment regimens contributes to the emergence of multi drug-resistant *Mycobacterium tuberculosis (Mtb)* strains, the causative agent of TB<sup>560</sup>. Additionally, it is estimated that about 2 billion people are latently infected with *Mtb* without showing signs of active disease<sup>560</sup>.

The immune system usually contains *Mtb* infection through the formation of granulomatous lesions<sup>561</sup>. However, immunosuppressed individuals such as those who have co-morbid human immunodeficiency virus (HIV) infection or diabetes mellitus have an impaired ability to control latent TB infection (LTBI)<sup>562</sup>, resulting in active disease and transmission. In fact, reactivation of LTBI is the number one cause of death in HIV co-infected individuals<sup>563,564</sup>. The gradual decline of CD4<sup>+</sup> T cells, the hallmark of HIV infection, is believed to be a major

contributing factor in LTBI reactivation<sup>561</sup>. CD4<sup>+</sup> T cells are a major source of interferon gamma (IFN-γ), a critical cytokine for TB control, and essential for the structural integrity of the granulomas<sup>565</sup>. However, the precise role CD4<sup>+</sup> T cells play in immunity to TB remains a matter of debate<sup>566,567</sup>. In this context, it is interesting that although anti-retroviral therapy largely restores CD4<sup>+</sup> T cell numbers, the increased risk for reactivation of LTBI is only partially diminished<sup>568</sup>, and reactivation of LTBI often occurs early after HIV infection<sup>569</sup>. Furthermore, in a macaque model of TB/HIV co-infection, suppression of LTBI reactivation was shown to be independent of CD4<sup>+</sup> T cells in at least one third of animals<sup>570</sup>. In addition, it was very recently demonstrated that a higher monocyte and macrophage turnover was responsible for LTBI reactivation in macaques co-infected with *Mtb* and simian immunodeficiency virus (SIV)<sup>571</sup>. Collectively, these findings further challenge the assumption that CD4<sup>+</sup> T cells are irreplaceable in TB.

The only licensed TB vaccine, Bacille Calmette-Guérin (BCG) is universally used. BCG efficiently prevents miliary and meningeal TB in children, but shows low efficacy against pulmonary TB in adults<sup>572</sup>, and hence does not prevent the transmission cycle<sup>573</sup>. Over the last decades several new TB vaccines have been developed with a few currently undergoing clinical trials<sup>574</sup>. The important role of IFN- $\gamma$ -secreting CD4<sup>+</sup> T cells in animal studies, has led to cognate activation and expansion of Mtb-specific CD4<sup>+</sup> T cells through the use of immunodominant Mtb antigens being the main strategy for many new TB vaccines under development<sup>575</sup>. However, the recent failures of the TB vaccine candidate MVA85A,<sup>576,577</sup> highlight the need to rethink TB vaccine design and to identify CD4-independent mechanisms that contribute to control of TB. Importantly, it has become increasingly clear that the immunological correlates of vaccine induced protection against Mtb are not fully understood and seem to differ between experimental TB vaccines<sup>566,567</sup>. BCG is administered intradermally in early childhood and most TB vaccine candidates in clinical trials are also administered intradermally<sup>578,579</sup>. However, a shortcoming of intradermal BCG administration is the development of weak memory lymphocyte responses, which lack mucosal homing chemokine receptors, such as CCR5 and CXCR3, that allow migration to the lung, the initial site of *Mtb* infection<sup>580</sup>. To match the route of vaccination to the route of natural infection, mucosal vaccination into the lung has attracted renewed interest<sup>568,581-583</sup>. It is now clear that vaccination directly into the respiratory tract (aerosol, i.n. and i.t.) generates more protective lung-residing memory T cells<sup>568,581-585</sup>.

Recombinant BCG strains and attenuated *Mtb* strains have received significant attention as potential replacement vaccines for BCG<sup>572,586</sup>. Live vaccines often elicit a broader immune response compared to protein-based formulations and do not require an adjuvant. The recombinant BCG  $\Delta ureC::hlv$  (VPM1002), and the attenuated *Mtb*  $\Delta phoP$   $\Delta fadD26$ (MTBVAC) are currently undergoing testing in various clinical trial stages<sup>587,588</sup>. Other experimental live recombinant BCG vaccines, such as BCGABCG1419c, BCG Azmp1 and BCG::ESX-1<sup>Mar</sup>, have shown promising results in animal models but have not yet reached human trials<sup>589-592</sup>. Similarly, attenuated *Mtb* strains, such as *Mtb*  $\Delta sigH^{584}$  and *Mtb*  $\Delta RD1$  $\Delta panCD^{593}$  showed increased protection, improved safety and better antigen-specific immune responses in various animal models. The live attenuated BCG-based vaccine candidate BCG $\Delta$ BCG1419c which lacks c-di-GMP phosphodiesterase gene *BCG1419c* was developed following the hypothesis that chronic mycobacterial infections and LTBI reproduce aspects of biofilm-formation, and contain different antigens compared to planktonic bacteria<sup>594,595</sup>. BCGABCG1419c showed improved protection against chronic Mtb infection in BALB/c mice and prevented reactivation from latent-like infection of B6D2F1 mice<sup>589</sup>, as well as improved protection against chronic Mtb infection of C57BL/6 mice, compared to parental BCG<sup>596</sup>. Recently, proteomic comparison of BCGABCG1419c has shown that in comparison to BCG, it slightly reduces its production of antigenic proteins such as PstS2, HbhA, CFP17, DnaK and 35 KDa antigen<sup>597</sup> and transcriptomic comparison of the same strains showed that BCGABCG1419c had reduced expression of groEL1, kasA, fas, fabD, acpM, and kasB, involved in mycolic acid biosynthesis, as well as reduced transcription of genes hspX, groEL2, and groES, which encode for antigenic chaperones<sup>596</sup>, globally contributing to a reduced inflammatory environment during chronic infection.

Several meta-analyses of human studies have found that BCG vaccination protects against active tuberculosis<sup>101,598-603</sup>. However, due to a lack of long-term human follow-up studies and the lack of studies regarding LTBI reactivation in the animal model that most closely resembles LTBI, the non-human primate, in the context of vaccination solely with BCG, it is currently unknown if BCG vaccination also impacts on the reactivation dynamics of LTBI in the context of HIV. Recent mathematical modelling data provided new evidence on the global prevalence of LTBI<sup>604</sup>, but fell short of estimating the role of BCG vaccination on LTBI reactivation. A very recent study also found no difference in the prevalence of LTBI in the UK, where the relative incidence between BCG vaccinated and naïve people was compared<sup>605</sup>. Another very recent report predicted the possible estimation of the role of BCG vaccination on reactivation

from LTBI, but the final outcome is yet to be reported<sup>606</sup>. In a statistically underpowered Taiwanese study the percentage of T-SPOT.TB positive, HIV-infected patients, was almost 50% less in people showing a BCG scar compared with those with no evidence of BCG vaccination, therefore suggesting BCG reduces reactivation from LTBI in a HIV setting<sup>607</sup>. Hence, in order to better understand the correlates of BCG-induced protection against LTBI reactivation, better models and a deeper understanding of the underlying immune response are required.

Non-human primate models for Mtb/SIV co-infection closely resemble human physiology, but are associated with substantial ethical, financial and logistical limitations<sup>419</sup>. We have recently described a new tractable mouse model to study reactivation of LTBI, following the loss of CD4<sup>+</sup> T cells similar to what occurs in HIV co-infection in humans<sup>425</sup>. In this model intradermally (i.d.) infected C57BL/6 mice contain Mtb within the local draining lymph nodes (LN) until depletion of CD4<sup>+</sup> cells occur, thereby mimicking the reactivation of LTBI following HIV infection. In contrast, Mtb-infected CD4-deficient mice and MHC-II deficient mice do not recapitulate the latent aspect of LTBI but rather only show exacerbated disease relative to C57BL/6 mice<sup>426</sup>. It was recently proposed that TB has characteristic features of lymphatic diseases and that pulmonary pathology may primarily serve disease transmission<sup>427</sup>. The hypothesis that *Mtb* persistence may occur in the lymphatics is supported by historical descriptions of TB cases, experimental models and observations of TB in non-human hosts<sup>427</sup>. This model is also supported by recent findings that *Mtb* persists in bone marrow stem cells<sup>608-</sup> <sup>611</sup>. Additional evidence for the importance of lymphatic persistence in LTBI has recently also been provided in high-profile NHP studies<sup>428,429</sup>. In this context, the robust mouse LTBI reactivation model presents a much-needed alternative to study LTBI compared to logistically challenging NHP models, and provides an opportunity to thoroughly investigate the importance of CD4<sup>+</sup> T cell-independent strategies for TB vaccination, which are likely to significantly contribute to the immune response elicited by a broadly protective vaccine.

Using this model, the present study examined (i) if reactivation of murine lymphatic LTBI following the loss of CD4<sup>+</sup> T cells can be prevented by prior BCG vaccination, (ii) if reactivation dynamics differ between vaccination routes, and (iii) if the genetically modified BCG $\Delta$ BCG1419c strain can reduce reactivation from lymphatic LTBI in this model.

# 3.3 Results

### 3.3.1 Vaccination profiling

To determine if BCG vaccination can prevent reactivation of lymphatic murine LTBI following the loss of CD4<sup>+</sup> cell, we vaccinated C57BL/6 mice with two different strains of BCG: BCG Pasteur (hereafter referred to as BCG) and a BCG Pasteur strain deficient in the gene *BCG1419c* (hereafter referred to as BCG $\Delta$ BCG1419c). BCG $\Delta$ BCG1419c vaccination reduced lung pathology and *Mtb* replication in 3 mouse models<sup>589,594,612</sup>, two of which resemble chronic infection, and the other one resembling reactivation from latent infection. Here, we further evaluated BCG $\Delta$ BCG1419c's potential as a vaccine candidate against infection in a model resembling reactivation of LTBI upon CD4 deprivation. Mice received the vaccines either as a parenteral subcutaneous (s.c.) injection into the tail base or directly into the lung via intratracheal (i.t.) vaccination (**Figure 3-1**). We reasoned that the increased pellicle production and *in vivo* persistence of BCG $\Delta$ BCG1419c would lead to increased memory T cell influx into the lung and subsequent reduction in the spread of *Mtb*.





Naïve C57BL/6 mice were vaccinated with BCG or BCGΔBCG1419c via the s.c. or i.t. route. 60 days after vaccination a group of mice was sacrificed and assessed for clearance of the vaccine strain, cellular immune responses, lung histology and serum cytokines/ chemokines. The remaining mice were infected i.d. with *Mtb* H37Rv in the ear dermis. At weekly intervals, mice received i.p. injections of a mAb against mouse CD4 (GK1.5) or PBS. On days 14, 28, 50 and 120 p.i mice were sacrificed. Ear-draining LNs, spleen and lung were assessed for numbers of viable *Mtb*, T cell populations by FACS, lung histology and serum cytokines. Unvaccinated mice without anti-CD4 treatment served as control for latent lymphatic TB and unvaccinated mice treated with anti-CD4 were used as control for reactivation of latent lymphatic infection and systemic spread. Abbreviations: BCG, Bacille Calmette–Guérin; *Mtb*, *Mycobacterium tuberculosis*; TB, tuberculosis; s.c., subcutaneous; i.t., intratracheal; i.p., intraperitoneal; i.d., intradermal

No weight loss or other adverse events were observed during the vaccination period in all groups. 60 days after vaccination we analysed bacterial clearance, immune cell composition, lung pathology and serum cytokine profiles. In all vaccination settings bacteria were efficiently cleared, and residual BCG colonies, close to the detection limit, were detected in only 5 out of 40 mice (**Figure 3-2A**). As previously shown<sup>585</sup>, i.t. vaccinations significantly increased the numbers of various T cell subsets in bronchioalveolar lavage fluid (BALF) and lung, including

CD44<sup>+</sup> memory cells and CD69<sup>+</sup>CD103<sup>+</sup> resident memory T cells (Figure 3-2B). The largest increase in T cell numbers was observed following i.t. BCGABCG1419c vaccination, with a 1.5 to 2.3-fold increase in BALF and a 2.8 to 5.4-fold increase in lungs in comparison to i.t. BCG (CD4<sup>+</sup>CD44<sup>+</sup> 4.03-fold p=0.0016; CD4<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup> 5.41-fold p=0.0012; CD8<sup>+</sup>CD44<sup>+</sup> 2.8-fold p=0.0048; CD8<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup> 3.24-fold p=0.0006). Subcutaneous vaccinations induced a modest increase in T cell numbers in spleen, BALF and lung (Figure S3-1A), and no differences in cellularity were detected in the inguinal LN (Figure S3-1A). We also assessed the level of cell infiltration into the lung following the different vaccination regimens by hematoxylin and eosin staining. Compared to naïve mice, the level of cell infiltration after s.c. vaccination was not increased (Figure 3-2C). Intratracheal vaccinations with both BCG strains led to a low but significant influx of immune cells into the lung which formed organized lymphoid structures, reminiscent of inducible Bronchus Associated Lymphoid Tissue (iBALT) (Figure 3-2C). Overall, low levels of circulating cytokines and chemokines were detected in all groups, with very few significant differences compared to unvaccinated animals (Figure 3-2D; Figure S3-1B). Although not reaching statistical significance, relative to s.c. vaccination i.t. vaccination led to reduced upregulation or even a downregulation of many circulating cytokines, including IL-10, IL-6, IL-22, IL-1β, IL-18 and IL-9 (Figure 3-2D). Collectively, these results demonstrate the safety of all BCG vaccination regimens and indicate a superior capacity of BCGABCG1419c to induce memory T cells following i.t. administration.



#### Figure 3-2: Immune profiling prior to Mtb infection.

Sixty days after vaccination with BCG or BCG $\Delta$ BCG1419c mice were assessed for: (**A**) Number of viable BCG in inguinal LN, spleen and lung; (**B**) Number of total CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup>(DN) and CD3<sup>-</sup>NKp46<sup>+</sup> cells (left plots), as well as numbers of total CD44<sup>+</sup>, CD44<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup> cells amongst CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells (right plots) in BALF and lung (**C**) lung immune cell infiltration; and (**D**) serum cytokines/ chemokines. Results are presented as individual data points (**A**, **C**), pooled data means  $\pm$  SEM (**B**), representative images (**C**) and as a heat map showing fold upregulation relative to naïve controls (**D**) from two pooled independent experiments (n = 8-10) mice per group. Statistical analyses: Oneway ANOVA per cell type followed by Dunnett's multiple comparisons test; significant differences relative to unvaccinated mice are indicated by asterisks: \* p<0.05; \*\* p<0.01; \*\*\*\* p<0.001; \*\*\*\* p<0.001. Original magnification H&E × 25. Abbreviations: BCG, Bacille Calmette–Guérin; s.c., subcutaneous; i.t., intratracheal; BALF, bronchioalveolar lavage fluid; LN, lymph node
# **3.3.2** BCG vaccination prevents systemic spread of reactivated lymphatic *Mtb* infection

We next assessed if BCG vaccination can prevent the progression from LTBI to active murine TB as a consequence of CD4<sup>+</sup> T cell depletion. C57BL/6 mice were infected with 100 cfu Mtb H37Rv in the ear dermis 60 days after vaccination and thereafter treated weekly with anti-CD4 mAb. Unvaccinated anti-CD4 mAb-treated mice were used as a positive control for reactivation of LTBI. Unvaccinated mice that did not receive anti-CD4 mAb served as a negative control in which *Mtb* remained contained in the LN. We assessed the bacterial burden in ear-draining LNs, spleen and lung at different time points (Figure 3-1). As previously reported<sup>425</sup>, unvaccinated untreated animals almost exclusively contained *Mtb* within the draining LNs of the infected ear with very limited systemic spread. In some animals few bacteria were detected in the spleen but almost never in the lung (Figure 3-3A-C, black bars). In unvaccinated animals treated with anti-CD4 mAb, Mtb not only multiplied significantly within the LNs over time but also exited the ear-draining LNs and spread to spleen and lung in all animals (Figure 3-3A-C, red bars). Prior vaccination with BCG or BCGABCG1419c significantly reduced replication of Mtb within the draining lymph nodes and led to a significantly reduced spread of the bacteria to the spleen (Figure 3-3B, grey and brown bars). Most importantly, all vaccination strategies prevented spread of *Mtb* to the lung following treatment with anti-CD4 mAb (Figure 3-3C, grey and brown bars). Particularly at 120 days after *Mtb* infection, unvaccinated animals that were treated with anti-CD4 contained an average of 100,000 bacteria in the lung, whereas vaccinated mice consistently showed CFU numbers below or near the detection limit. No consistent patterns were observed, with both BCG strains and vaccination routes being equally protective. However, particularly during later time points (d120), vaccination with BCGABCG1419c led to very low numbers of detectable Mtb in spleen and lung, and also in the LN after i.t. vaccination (Figure 3-3A-C, brown bars).





Sixty days after vaccination with BCG or BCG $\Delta$ BCG1419c mice were infected i.d. with 1×10<sup>2</sup> *Mtb* H37Rv. At weekly intervals, mice received an i.p. injection with a mAb against mouse CD4 (GK1.5) or PBS. On days 14, 28, 50 and 120 p.i mice were sacrificed and the ear-draining LNs, spleen and lung were assessed for viable bacteria (A-C). Sera and lung sections from 120 days p.i. were also assessed for cytokines/chemokines (**D**) and immune cell infiltration (**E**), respectively. Results are presented as pooled data means ± SEM (A-C), individual data points and representative images (**E**) and as a heat map showing fold upregulation relative to unvaccinated untreated controls (**D**) from two pooled independent experiments (n = 8-10 mice per group). Statistical analyses: One-way ANOVA per time point followed by Dunnett's multiple comparisons test; significant differences relative to unvaccinated anti CD4-treated mice are indicated by asterisks: \* p<0.05; \*\* p<0.01; \*\*\* p<0.001; \*\*\*\* p<0.0001; dottet line in **A-C** represents CFU detection limit. Original magnification H&E × 25. Abbreviations: BCG, Bacille Calmette–Guérin; s.c., subcutaneous; i.t., intratracheal; LN, lymph node; mAb, monoclonal antibody

Consistent with low numbers of detectable bacteria in the lung in BCG vaccinated groups, vaccination also significantly reduced levels of pro-inflammatory cytokines, such as MCP-3, IL-6, IL-18, Eotaxin and RANTES relative to unvaccinated, anti-CD4 mAb-treated controls (Figure 3-3D; Figure S3-2). While circulating serum levels of IL-10 and IL-22 were reduced following i.t. vaccination (Figure 3-2D; Figure S3-1B), these cytokines were upregulated in i.t. vaccinated animals following *Mtb* infection compared to unvaccinated and s.c. vaccinated mice (Figure 3-3B; Figure S3-2). The levels of immune cell infiltration into the lungs of s.c. vaccinated mice were comparable to unvaccinated untreated mice (Figure 3-3E). In contrast, unvaccinated mice that had received anti-CD4 mAb showed significant and large-scale lung immunopathology and cell infiltration (Figure 3-3E). Intratracheally vaccinated animals maintained the level of immune cell infiltration that was observed 60 days after vaccination around the bronchioli, despite showing very low or even undetectable levels of CFU in the lung. The overall infiltration scores of i.t. vaccinated mice were not significantly different to unvaccinated anti-CD4-treated mice. However, in contrast to the diffuse and widespread infiltration seen in anti-CD4-treated unvaccinated animals, the infiltration in i.t. vaccinated animals resembled well organized and localized iBALT clusters seen prior to Mtb infection (Figure 3-3E and 3-2C). Together these data demonstrate that BCG vaccination prevents reactivation of latent lymphatic murine TB regardless of the BCG strain used for vaccination. The disparate cytokine levels and immune cell infiltration patterns also suggest that prevention of LTBI reactivation following s.c. and i.t. vaccination may depend on different mechanisms.

#### 3.3.3 Vaccine-induced prevention of reactivation is independent of CD4<sup>+</sup> T cells

Given that anti-CD4 treatment in unvaccinated mice (**Figure 3-3A-C**, red bars) led to reactivation of LTBI, we also investigated the efficiency of CD4<sup>+</sup> T cell depletion. In all groups, anti-CD4 mAb treatment led to almost complete depletion of CD4<sup>+</sup> T cells from eardraining lymph nodes as well as from the lung parenchyma (**Figure 3-4A-D**). Depletion efficacy progressively increased from day 14 (**Figure S3-3A, B**), and by day 120 after infection only 7 out of 50 mice showed low residual levels of CD4<sup>+</sup> T cells in lymph nodes and lung (**Figure 3-4C, D**). In line with effective depletion of CD4<sup>+</sup> T cells, the frequency of CD8<sup>+</sup> T cells significantly increased in LN and lung of all anti-CD4-treated mice (**Figure 3-4E, F; Figure S3-3C, D**). However, this proportional increase was initially not accompanied by a numerical increase, because at days 14, 28 and 50 p.i. total CD8<sup>+</sup> T cell numbers in LN and lung were not statistically significant between groups (**Figure S3-3E, F**). Only the anti CD4-treated unvaccinated group, the s.c. BCG group and the s.c. BCGΔBCG1419c group reached statistical significance in the lung but not the LN at day 120 p.i. (Figure 3-4G, H). Similarly, while CD44<sup>+</sup> memory CD8<sup>+</sup> T cells proportionally increased following anti-CD4 mAb treatment (Figure 3-4I, J; Figure S3-3G, H), statistically significant numerical increases were only observed at day 120 p.i. in the lung (Figure 3-4K, L; Figure S3-3I, J). These results suggest that depletion of CD4<sup>+</sup> T cells does not lead to a numerical replacement by CD8<sup>+</sup> T cells to control infection in the LN and lung. An additional statistical comparison between unvaccinated anti-CD4-treated mice with anti-CD4-treated vaccinated groups (red asterisks in Figure 4 and Supplementary Figure 3), revealed that the proportional increase in CD8<sup>+</sup> T cells in the lung of vaccinated mice was lower in almost all vaccination groups at days 14, 28 and 50 after *Mtb* infection (Figure S3-3D). These differences were not observed at 120 days after challenge and did not translate into statistically significant numerical differences in total CD8<sup>+</sup> T cell numbers (Figure S3-3F) or memory CD8<sup>+</sup> T cell numbers (Figure S3-3J) for most vaccination groups. Compared to unvaccinated anti-CD4-treated mice, only i.t. BCGABCG1419c vaccinated mice showed significantly reduced numbers in total CD8<sup>+</sup> (Figure 3-4H) and memory CD8<sup>+</sup> T cells numbers (Figure 3-4L) in the lung 120 days after Mtb challenge. Furthermore, when we assessed memory CD8<sup>+</sup> T cells for the expression of CD62L, CD69 and CD103 to compare frequencies of effector-, central- and resident memory T cells, no obvious differences between the vaccination groups and unvaccinated control groups were observed. However, the increase in resident memory T cells that was only induced by i.t. vaccination (Figure 3-2B) was maintained following *Mtb* challenge in the lung (Figure S3-4A). Overall these data unambiguously demonstrate that BCG vaccination prevents LTBI reactivation without a requirement for CD4<sup>+</sup> T cells, and suggest that numerical changes in CD8<sup>+</sup> T cells are unlikely to mediate the prevention of LTBI reactivation in this model.



#### Figure 3-4: Vaccine-induced prevention of reactivation is independent of CD4+ T cells.

(A, B) Representative FACS plots showing depletion of CD4<sup>+</sup> T cells in LN and Lung (gated on CD3<sup>+</sup> cells) following administration of anti-CD4 mAb. (C, D) Frequencies of CD3<sup>+</sup>CD4<sup>+</sup> cells in LN (C) and lung (D) at 120 days following *Mtb* infection. (E, F) Frequencies of CD3<sup>+</sup>CD8<sup>+</sup> cells in LN (E) and lung (F) at 120 days following *Mtb* infection. (G, H) Total numbers of CD3<sup>+</sup>CD8<sup>+</sup> cells in LN and lung at 120 days following *Mtb* infection. (I, J) Frequencies of CD3<sup>+</sup>CD4<sup>+</sup> cells in LN and lung at 120 days following *Mtb* infection. (K, J) Total number of CD3<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup> cells in LN and lung at 120 days following *Mtb* infection. Results are presented as representative FACS plots (A, B) or individual data points (C-L) from two pooled independent experiments (n = 8-10 mice per group). Statistical analyses: One-way ANOVA followed by Dunnett's multiple comparisons test; significant differences relative to unvaccinated mice are indicated by red asterisks; statistical differences relative to unvaccinated anti-CD4-treated mice are indicated by red asterisks: \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.0001; Abbreviations: BCG, Bacille Calmette–Guérin; s.c., subcutaneous; i.t., intratracheal; LN, lymph node; mAb, monoclonal antibody

### 3.4 Discussion

Using a tractable mouse model, we provide compelling evidence that immune control of reactivation of latent lymphatic *Mtb* infection can be independent of vaccine-induced CD4<sup>+</sup> T cells. Treatment of latently infected mice with anti-CD4 mAb caused LTBI reactivation and systemic spread of *Mtb* to spleen and lung, mimicking the rapid progression from LTBI to TB in HIV<sup>+</sup> individuals<sup>425</sup>. In contrast, prior vaccination with BCG or BCG $\Delta$ BCG1419c prevented reactivation of *Mtb* from the ear-draining lymph nodes and systemic spread, regardless of the route of vaccine delivery. As most mice were depleted of CD4<sup>+</sup> T cells, our results unambiguously demonstrate that vaccine-induced non-CD4 T cell responses are sufficient to control latent lymphatic *Mtb* infection. Furthermore, we provide evidence that the experimental TB vaccine BCG $\Delta$ BCG1419c is safe and induces more memory T cells in BALF and lung tissue following i.t. vaccination. Collectively, these results further highlight the incomplete understanding about what constitutes immunity in TB and why BCG only efficiently prevents some forms of TB but not others<sup>566,567</sup>.

As expected, the frequency of CD8<sup>+</sup> T cell in the infected lymph nodes and lungs increased following the depletion of CD4<sup>+</sup> T cells. However, the total number of CD8<sup>+</sup> T cells only significantly increased in the lungs of unvaccinated anti-CD4-treated mice and s.c. vaccinated mice at 120 days after *Mtb* infection. Similarly, while the frequency of CD44<sup>+</sup> memory CD8<sup>+</sup> T cells increased after depletion of CD4<sup>+</sup> T cells, the total numbers of these cells were only significantly increased at 120 days after Mtb infection in the lung of unvaccinated anti-CD4treated mice, s.c. vaccinated mice and i.t. BCG vaccinated mice. Importantly, i.t. vaccination with BCGABCG1419c did not significantly increase CD8<sup>+</sup> T cell populations, but even significantly reduced CD8<sup>+</sup> T cell populations relative to unvaccinated anti-CD4-treated mice, despite providing robust long-term protection against systemic spread of Mtb. These results suggest that protection from reactivation is unlikely to be exclusively mediated by an increase in CD8<sup>+</sup> T cell populations, but may perhaps rely on BCG $\Delta$ BCG1419c's improved capacity to stimulate local CD8<sup>+</sup> IFN  $\gamma^+$  T lymphocytes in response to *Mtb* antigens, as previously reported<sup>589</sup>. This is consistent with our previous study in which we had investigated if the boost of memory CD8<sup>+</sup> T cells, DN T cells and NK cells with IL-2/anti-IL-2 complexes can reverse the reactivation of lymphatic LTBI. Although IL-2/anti-IL-2 complex treatment significantly expanded these immune cell populations in LN, spleen and lung, it did not prevent the systemic spread of *Mtb* after CD4<sup>+</sup> T cells were depleted<sup>425</sup>. It was also recently shown that CD8<sup>+</sup> T cells fail to recognize *Mtb*-infected macrophages, due to bacterial-induced decoy mechanism, using distinct immunodominant *Mtb* antigens in an *in vitro* model<sup>613</sup>. Further studies are needed to investigate the role of antigen-specific CD8<sup>+</sup> T cells and their function in localized IFN- $\gamma$  production in this model. On the other hand, it could be that such protection from reactivation of lymphatic LTBI could be mediated by activated macrophages, which were shown to be more abundant in another model of reactivation from latent-like infection upon corticosteroid treatment, as opposed to IFN $\gamma^+$  T cells<sup>589</sup>. An increased turnover rate of tissue macrophage was also suggested to be important in TB reactivation in rhesus macaques<sup>571</sup>. Further studies will be required to formally confirm or to rule out these hypotheses.

It was beyond the scope of this study to investigate which immune mechanisms contain Mtb infection in the absence of CD4<sup>+</sup> T cells. Hence, we did not further investigate humoral immunity, B cell characteristics or innate (trained) immune responses. The importance of such immune responses in TB has recently received renewed attention<sup>614,615</sup>. In particular the distinct antibody glycosylation pattern identified in human LTBI<sup>616</sup> has highlighted a potentially important role for antibody-mediated effector function in controlling latent infection. Furthermore, there is increasing evidence that *Mtb* exposure generates antibody isotypes in humans that can inhibit mycobacterial infection<sup>617</sup>, particularly in previously uninfected individuals and individuals with LTBI<sup>618</sup>. It is also interesting to note that patients with active TB appear to have dysfunctional circulating B cells, which regain function following successful treatment<sup>619</sup>. In support of our study, it was also recently demonstrated that reactivation of LTBI in a macaque model of TB/HIV co-infection was independent of CD4<sup>+</sup> T cells in at least one third of all animals<sup>570</sup>. Collectively these results point towards an important role of B cell-mediated immunity in LTBI control as well as prevention of Mtb infection. Our study extends these findings, and it is hence tempting to speculate that BCG vaccination may prevent reactivation of LTBI via B cell-mediated or trained innate immunity. The tractable, reproducible and widely affordable mouse model presented here, will allow to study the contributions of these mechanisms on the dynamics of LTBI reactivation in future studies.

The recombinant BCG $\Delta$ BCG1419c has previously been shown to protect against *Mtb* challenge in chronic TB models<sup>589,596</sup>. Our study also provides evidence that BCG $\Delta$ BCG1419c induces equivalent protection in this model of LTBI reactivation compared to conventional BCG against dissemination to the lungs, and improved reduction of *Mtb* replication in LN and spleens at the latest time point evaluated in this work, 120 days post-infection (**Figure 3-3A**,

C). Intratracheal administration of BCG $\Delta$ BCG1419c induced significantly higher numbers of memory T cells in the lung, including cells with resident memory phenotype. Mucosal vaccination with BCG $\Delta$ BCG1419c also induces significantly higher levels of organized lymphoid structures in the lung, similar to iBALT. Although these findings do not explain why subcutaneous vaccination also prevents systemic spread of *Mtb* in this model, there is increasing evidence that lymph node architecture also changes significantly towards a B cell-dominated structure following infection<sup>620</sup>. Taken together, it is possible that both s.c. and i.t. BCG vaccinations protect against reactivation of LTBI by anatomically distinct but functionally similar mechanisms that are geared towards expansion of B cells. Further studies will have to investigate these hypotheses.

HIV infection in humanized mice and SIV infection in NHPs have shown to preferentially deplete highly protective CD4<sup>+</sup> T<sub>RM</sub> cells compared to blood and alveolar cells <sup>621</sup>. Although our data indicate an efficient depletion of CD4<sup>+</sup> T cells in lung, spleen and LNs following anti-CD4 treatment, it has recently been shown that skin T<sub>RM</sub> cells were resistant to in vivo antibodymediated cell depletion <sup>622</sup>. We cannot therefore entirely exclude the potential role of skin CD4<sup>+</sup> T<sub>RM</sub> cells that have not been effectively depleted by the anti-CD4 mAbs in terms of preventing the dissemination of *Mtb* from the ears of the animals thus this needs to be addressed in future studies. In addition, future investigations should also focus on determining whether a LTBI model in genetically modified mouse strains lacking particular immune cell subsets can be established. A combination of different KO mouse strains and different vaccines may ultimately reveal which cell type contains *Mtb* in the absence of CD4<sup>+</sup> T cells. These studies should be accompanied by detailed, high-dimensional imaging analyses of the immune cell compositions in infected lymph nodes, spleen and lung to dissect whether containment of *Mtb* largely occurs in the infected lymph node or via preferential killing by vaccine-induced immune cell subsets in the spleen and/or lung. The time-dependent spread of *Mtb* from LN to spleen and lung following reactivation with anti-CD4 suggests the presence of highly coordinated tissue-specific immune responses that may involve macrophages and other antigen presenting cells. Additionally, the absence of *Mtb* CFU in the lung of vaccinated animals may also suggest that the spread of *Mtb* from the lymphatics to the lung is interrupted, and that vaccine-induced containment predominantly occurs in the infected lymph nodes and the spleen. All of these hypotheses will require further investigation.

In summary, our results provide compelling evidence that BCG vaccination protects against reactivation of LTBI independently of vaccine-induced  $CD4^+$  T cells. Our findings suggest that protection from reactivation may be independent of  $CD8^+$  T cell expansion and suggest a potential role for B cells, antibody and/or trained immunity in preventing reactivation. Finally, these results also underpin the importance of our new small animal model of LTBI to gain new insights into the correlates of BCG-induced immunity against *Mtb*.



## 3.5 Supplementary Figures and Tables for Chapter 3

#### Figure S3-1: T cell numbers, serum cytokine and chemokine levels prior to Mtb infection.

(A) Number of total CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> (DN) and CD3<sup>-</sup> NKp46<sup>+</sup> cells (left plots), as well as numbers of total CD44<sup>+</sup>, CD44<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup> cells amongst CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> cells (right plots) in spleen and inguinal LN 60 days after vaccination with BCG or BCG $\Delta$ BCG1419c. (B) Levels of serum cytokines and chemokines at 60 days after vaccination. Results are presented as pooled data means  $\pm$  SEM from two pooled independent experiments (n = 8-10 mice per group). Statistical analyses: One-way ANOVA per analyte followed by Dunnett's multiple comparisons test; significant differences relative to unvaccinated mice are indicated by asterisks: \* p < 0.05; \*\* p < 0.01; \*\*\* p < 0.001; \*\*\*\* p < 0.001. Abbreviations: BCG, Bacille Calmette-Guérin; s.c., subcutaneous; i.t., intratracheal; (Supplementary to Fig. 3-2)



#### Figure S3-2: Serum cytokine and chemokine levels 120 days after Mtb infection.

(A) Levels of serum cytokines and chemokines at 120 days after Mtb infection. Results are presented as pooled data means  $\pm$  SEM from two pooled independent experiments (n = 8-10 mice per group). Statistical analyses: One-way ANOVA per analyte followed by Dunnett's multiple comparisons test; significant differences relative to unvaccinated mice are indicated by asterisks: \* p < 0.05; b.d. below detection limit. Abbreviations: BCG, Bacille Calmette-Guérin; s.c., subcutaneous; i.t., intratracheal; mAb, monoclonal antibody (Supplementary to Figure 3-3)



#### Figure S3-3: CD8<sup>+</sup> T cell profiling after Mtb infection.

(A, B) Frequencies of CD3<sup>+</sup>CD4<sup>+</sup> cells in LN (A) and lung (B) at 14, 28 and 50 days following *Mtb* infection. (C, D) Frequencies of CD3<sup>+</sup>CD8<sup>+</sup> cells in LN (C) and lung (D) at 14, 28 and 50 days following *Mtb* infection. (E, F) Total numbers of CD3<sup>+</sup>CD8<sup>+</sup> cells in LN and lung at 14, 28 and 50 days following *Mtb* infection. (G, H) Frequencies of CD3<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup> cells in LN and lung at 14, 28 and 50 days following *Mtb* infection. (I, J) Total number of CD3<sup>+</sup>CD8<sup>+</sup>CD44<sup>+</sup> cells in LN and lung at 14, 28 and 50 days following *Mtb* infection. Results are presented as individual data points from two pooled independent experiments (n = 8-10 mice per group). Statistical analyses: One-way ANOVA per time point followed by Dunnett's multiple comparisons test; significant differences relative to unvaccinated mice are indicated by red asterisks: \* p<0.05; \*\* p<0.01; \*\*\* p<0.001; \*\*\*\* p<0.0001. Abbreviations: BCG, Bacille Calmette–Guérin; s.c., subcutaneous; i.t., intratracheal; LN, lymph node; mAb, monoclonal antibody (Supplementary to Figure 3-4)





(A) Frequencies of CD44<sup>+</sup>CD62L<sup>-</sup>, CD44<sup>+</sup>CD62L<sup>+</sup> and CD44<sup>+</sup>CD69<sup>+</sup>CD103<sup>+</sup> cells amongst CD8<sup>+</sup> T cells. Results are presented as pooled data means from two pooled independent experiments (n = 8-10 mice per group). Statistical analyses: Abbreviations: BCG, Bacille Calmette–Guérin; s.c., subcutaneous; i.t., intratracheal; LN, lymph node; mAb, monoclonal antibody (Supplementary to **Figure 3-4**)

## Table S3-1: P-values for CFU results presented in Figure 3-3A-C.

A one-way ANOVA per time point followed by Tukey's multiple comparisons test was performed for CFU results shown in Figure 3A. The mean of each group was compared to the mean of every other group and P values for each combination and time point are shown. (Supplementary to **Figure 3-3**)

Δ1419c i.t.

LN d14	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	∆1419c i.t.
unvacc.		0.758	0.9516	0.998	0.6265	0.9892
anti CD4			0.9969	0.9409	>0.9999	0.3756
BCG s.c.				0.9978	0.9823	0.6718
BCG i.t.					0.8663	0.8988
∆1419c s.c.						0.2637
Δ1419c i.t.						

LN

LN d28	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	∆1419c i.t.
unvacc.		0.498	0.9355	0.6945	0.568	0.5537
anti CD4			0.0117	0.0026	0.0014	0.0013
BCG s.c.				0.9952	0.979	0.976
BCG i.t.					>0.9999	>0.9999
Δ1419c s.c.						>0.9999
∆1419c i.t.						

LN d50	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	Δ1419c i.t.
unvacc.		0.0128	0.9986	0.9632	0.9812	0.945
anti CD4			0.0559	0.0015	0.0894	0.115
BCG s.c.				0.8532	0.9998	0.9976
BCG i.t.					0.68	0.544
Δ1419c s.c.						>0.9999
Δ1419c i.t.						

LN d120	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	Δ1419c i.t.
unvacc.		0.0351	0.9548	0.8294	0.9902	0.2145
anti CD4			0.0041	0.3843	0,1696	0.0001
BCG s.c.				0.3297	0.7149	0.7568
BCG i.t.					0.9935	0.009
∆1419c s.c.						0.0669
Δ1419c i.t.						

Spreen								
PL d14	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	Δ1419c i.t.		
invacc.		0.9928	0.9747	0.4905	0.8973	0.9999		
inti CD4			0.7759	0.8309	0.5897	0.9996		
BCG s.c.				0.1356	0.9996	0.9162		
BCG i.t.			-		0.0682	0.6508		
1419c s.c.						0.7816		

SPL d28	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ14 <b>19c</b> s.c.	Δ1419c i.t.
unvacc.		0.0049	0.9614	0.4416	>0.9999	>0.9999
anti CD4			0.0457	0.3687	0.0025	0.0031
BCG s.c.				0.9067	0.8959	0.9219
BCG i.t.					0.3107	0.3511
∆1419c s.c.						>0.9999
Δ1419c i.t.						

SPL d50	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	Δ1419c i.t.
unvacc.		0.0001	0.9993	0.1203	0.945	0.8257
anti CD4			0.0001	0.0417	0.0003	0.0006
BCG s.c.				0.0781	0.8427	0.6718
BCG i.t.					0.5751	0.728
∆1419c s.c.						0.9997
Δ1419c i.t.						

SPL d120	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	Δ1419c i.t.
unvacc.		0.0001	0.3001	0.0902	0.9905	0.9373
anti CD4			0.0695	0.1481	0.001	0.0012
BCG s.c.				0.9969	0.6876	0.8044
BCG i.t.			2		0.3488	0.4524
∆1419c s.c.						0.9997
∆1419c i.t.						

Lung

Lung d14	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	Δ1419c i.t.
unvacc.		0.9095	0.4945	0.8678	0.4945	0.9909
anti CD4			0.9747	>0.9999	0.9747	0.9982
BCG s.c.				0.9873	>0.9999	0.8478
BCG i.t.					0.9873	0.9946
Δ1419c s.c.						0.8478
Δ1419c i.t.						

Lung d28	unvacc.	anti CD4	BCG s.c.	BCG i.t.	Δ1419c s.c.	Δ1419c i.t.
unvacc.		0.9038	0.6787	0.9749	0.9038	0.6787
anti CD4			0.9977	0.4853	>0.9999	0.9977
BCG s.c.				0.2429	0.9977	>0.9999
BCG i.t.					0.4853	0.2429
Δ1419c s.c.						0.9977
Δ1419c i.t.						

Lung d50	unvacc.	anti CD4	BCG s.c.	BCG i.t.	∆1419c s.c.	Δ1419c i.t.
unvacc.		0.0042	0.9995	0.9906	0.6839	0.6588
anti CD4			0.003	0.0283	0.0001	0.0001
BCG s.c.				0.9495	0.8879	0.8774
BCG i.t.					0.3468	0.3189
Δ1419c s.c.						>0.9999
Δ1419c i.t.						

Lung d120	unvacc.	anti CD4	BCG s.c.	BCG i.t.	∆1419c s.c.	Δ1419c i.t.
unvacc.		0.0001	>0.9999	0.9839	0.9957	0.9974
anti CD4			0.0001	0.0001	0.0001	0,0001
BCG s.c.				0.986	0.9978	0.8479
BCG i.t.			]		0.8479	0.8529
Δ1419c s.c.						>0.9999
Δ1419c i.t.						

Spleen

# **Chapter 4**

# Mucosal delivery of ESX-1-expressing BCG strains provides superior immunity against tuberculosis in murine type 2 diabetes

This chapter contains the manuscript titled 'Mucosal delivery of ESX-1-expressing BCG strains provide superior immunity against tuberculosis in murine type 2 diabetes' has been published in *Proceedings of the National Academy of Sciences U. S. A.* on 10<sup>th</sup> August 2020. (DOI: 10.1073/pnas.2003235117)

#### 4.1 Abstract

Tuberculosis (TB) claims 1.5 million lives per year. This situation is largely due to the low efficacy of the only licensed TB vaccine, Bacille Calmette Guérin (BCG) against pulmonary TB. The metabolic disease type 2 diabetes (T2D) is a risk factor for TB and the mechanisms underlying increased TB susceptibility in T2D are not well understood. Furthermore, it is unknown if new TB vaccines will provide protection in the context of T2D. Here we used a diet-induced murine model of T2D to investigate the underlying mechanisms of TB/T2D comorbidity and to evaluate the protective capacity of two experimental TB vaccines in comparison to conventional BCG. Our data reveal a distinct immune dysfunction that is associated with altered lung and fecal microbiomes and diminished recognition of mycobacterial antigens in T2D. Additionally, we provide compelling evidence that mucosal delivery of recombinant BCG strains expressing the *Mycobacterium tuberculosis* (*Mtb*) ESX-1 secretion system (BCG::RD1 and BCG::RD1 ESAT-6  $\Delta$ 92-95) are safe and confer superior immunity against aerosol *Mtb* infection in the context of T2D. Our findings suggest that remarkable anti-TB immunity by these recombinant BCG strains is achieved via augmenting the numbers and functional capacity of antigen presenting cells in the lungs of diabetic mice.

### 4.2 Background

Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis* (*Mtb*) and is the leading infectious cause of death globally. Approximately 10 million new TB cases were reported in 2018 with a further 1.7 billion people worldwide latently infected and at risk of reactivation <sup>8</sup>. Despite recent advances in diagnostics, treatment options and control measures, TB still kills an estimated 1.5 million people each year<sup>8</sup>. Reactivation of latent TB infection (LTBI) is strongly associated with comorbid immunosuppressing conditions, most notably human immunodeficiency virus (HIV) co-infection/AIDS and diabetes mellitus (DM)<sup>488</sup>. It is now recognized that the influence of DM, particularly type 2 diabetes (T2D) on TB burden is greater than HIV co-infection, because of its higher prevalence (~ 463 million people currently live with DM and the numbers are expected to escalate to 700 million by 2045<sup>436</sup>), with the majority of diabetics living in TB endemic countries. Based on recent meta-analyses, individuals with DM have a 3- to 4-fold increased risk of developing TB while ~16% of TB patients have comorbid DM<sup>18,20</sup>. Furthermore, the risk of reactivation of LTBI is significantly increased in TB/T2D comorbid patients<sup>492</sup>. TB/T2D comorbidity is not limited to low- to

middle-income countries but also exists in developed nations. As a result, TB/T2D comorbidity poses a significant challenge to the global eradication of TB.

Although the mechanisms underlying this increased susceptibility to TB are not well understood, multiple animal models of DM, including T2D, show defective innate immune recognition<sup>518</sup> and delayed adaptive immune-priming<sup>525</sup> relative to standard models. However, the majority of these animal models lack many features of T2D. We have recently described a robust diet-induced animal model for T2D encompassing the cardinal features of human T2D such as obesity, glucose intolerance, chronic inflammation, hyperinsulinemia, progressive insulin resistance and adipocyte and glomerular hypertrophy<sup>543</sup>. Using this model, we demonstrated increased bacterial burden, lung immunopathology and greater mortality following infections with *M. fortuitum*<sup>518</sup> and *M. bovis* BCG<sup>623</sup>. However, the precise defects that predispose the diabetic lung to TB disease remain unknown.

While the immunological correlates of TB protection are not well defined, the role of CD4<sup>+</sup> T cells in *Mtb* immunity is well-established in animal models and TB patients. Depletion of Th1 cells results in early disease reactivation from LTBI as seen in HIV patients<sup>203</sup>. However, accumulating evidence suggests a substantive role for CD4<sup>+</sup> T cell-independent protective immunity<sup>429</sup>. For example, we and others have recently shown that vaccine-induced CD4<sup>+</sup> T cells are not necessary to prevent the reactivation of LTBI in murine<sup>624</sup> and non-human primate (NHP) models<sup>429</sup>. Understanding which immune responses truly correlate with protection will be critical for the development of an effective TB vaccine. Bacille Calmette Guérin (BCG), the only approved TB vaccine to date does not provide sufficient protection against pulmonary TB in adults<sup>101</sup>. Current experimental TB vaccine strategies include: boosting BCG with improved and more immunogenic recombinant BCG (rBCG) strains; live attenuated Mtb vaccines or; subunit vaccines that are safe to use in immunocompromised individuals<sup>100</sup>. There is also renewed interest in intravenous (i.v.)<sup>625</sup> and mucosal delivery of TB vaccines, including BCG, primarily due to the increased protection afforded by pulmonary resident memory T cells  $(T_{RM})$ <sup>266</sup>. rBCG strains engineered to incorporate immunodominant *Mtb* regions, such as the virulence associated ESX-1 locus, cytokines, toxin-derived antigens and genes important for antigen presentation enhance and broaden the vaccine-induced immune response<sup>586</sup>. Furthermore, strategies that allow the vaccine strain to reach the cytosol via the incorporation of phagosome perforating molecules, such as the ESX-1 system<sup>48</sup> or listeriolysin (Hly) from *Listeria monocytogenes*<sup>626</sup> afford superior protection against an *Mtb* challenge. A number

of rBCG strains including BCG  $\Delta ureC::hly$  (VPM1002), which secretes membrane-perforating Hly are currently undergoing clinical trials<sup>586</sup>. However, whether these novel live-recombinant vaccine candidates will be safe and efficacious in the context of T2D is not known.

The goal of global eradication of TB by 2050 requires an effective, safe vaccine that works in all individuals vulnerable to develop TB disease, including those with diabetes. Using our dietinduced murine model of T2D, here we investigated the mechanistic basis of TB/T2D comorbidity and evaluated the safety and efficacy of experimental rBCG strains. We analyzed the lung microbiome profiles as well as the gene expression patterns in control and T2D mice to investigate the impact of T2D on lung immunity that could potentially render diabetic animals more vulnerable to *Mtb* infection. We compared the conventional BCG Pasteur strain with rBCG strains that secrete immunodominant ESX-1 antigens derived from the *Mtb* "region of difference 1" (RD1). Our findings demonstrate that perturbations in gut microbiomes in T2D are associated with altered functional capacity of lung immune cells. Most significantly, we found that mucosal administration of ESX-1-containing rBCG strains not only offers superior safety in the context of T2D but also confers outstanding protection against *Mtb* aerosol challenge. Immunological analyses indicate that rBCG-mediated anti-TB immunity was achieved via augmenting the anti-mycobacterial function of lung innate antigen presenting cells (APCs).

## 4.3 Results

### 4.3.1 Murine T2D mimics increased human susceptibility to aerosol *Mtb* infection

To induce murine T2D, male C57BL/6 mice were subjected to extended feeding of energy dense diet (EDD; Figure 4-1A) as shown previously<sup>543</sup>. Prior to diet intervention the body mass of mice was comparable between standard rodent diet (SD)-fed age-matched non-diabetic control and EDD-fed T2D groups (SD,  $17.94\pm0.1824$  g vs EDD,  $18.01\pm0.3365$  g; mean $\pm$ SEM, *P*=0.8620). After 30 weeks of diet intervention, mice fed with SD or EDD demonstrated an overall body weight increase of 83.55% and 152.24%, respectively. Body weight was significantly greater in the EDD group compared to the SD group (SD,  $32.93\pm0.5234$  g vs EDD,  $45.43\pm0.5604$  g, *P*<0.0001; Figure 4-1B). In addition, mice fed the EDD had significantly higher fasting blood glucose (SD,  $6.476\pm0.3$  mmol/L vs EDD,  $10.11\pm0.2981$  mmol/L, *P*<0.0001; Figure 4-1C) and impaired glucose tolerance as reflected by higher area under the curve (AUC) values (SD,  $1398\pm64.13$  vs EDD,  $2203\pm62.26$ , *P*<0.0001; Figure 4-1D); key metabolic features associated with the development of T2D.

To determine if the EDD-fed T2D mice are more prone to *Mtb* infection, we exposed T2D and control mice to a very-low dose (10-20 CFU) of *Mtb* H37Rv. There was no difference in lung *Mtb* burden between control and T2D mice at 1 day after *Mtb* challenge (Figure 4-1E), indicating that bacterial inhalation is not affected by T2D and all mice received a comparable dose. At 45 days after *Mtb* challenge however, T2D mice displayed significantly higher lung and spleen CFU loads (Figure 4-1F) accompanied by increased pathological damage to the lung tissue (Figure 4-1G and S4-1). Collectively, these data demonstrate that the diet-induced murine model of T2D mimics the cardinal features of human T2D and the increased susceptibility to aerosol *Mtb* infection, further confirming the appropriateness of this model to study TB/T2D comorbidity.



Figure 4-1: *Murine T2D mimics increased human susceptibility to aerosol Mtb infection.* (A) 4-6 weeks old C57BL/6 male mice were fed with EDD and SD (control mice) for 30 weeks to induce murine T2D. After the dietary intervention mice were assessed for (B) body weight, (C) fasting blood glucose levels and (D) glucose tolerance. (A) T2D confirmed naïve and control C57BL/6 mice were infected with very-low dose of aerosol *Mtb* H37Rv (10-20 CFUs). (E) One day p.i. 5 mice from each group sacrificed to confirm the initial infectious dose. 45 days p.i., infected lungs and spleens were assessed for (F) viable *Mtb* and (G) % of lung affected. Results are presented as (B-G) individual data points, (D) pooled data means and (G) representative images (Magnification, 25x; Scale bar, 500 µm) from (B) 50, (C, D) 25 and (E-G) 5 mice per group. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.001, by unpaired 2-tailed Student's *t*-test. Data are means  $\pm$  SEM.

## 4.3.2 Compositional changes in the lung and gut microbiota following *Mtb* infection

The resident microbiota has a pivotal role in the onset of T2D and its plethora of complications while perturbed microbiome compositions and altered immune responses have both been implicated in many metabolic diseases<sup>373</sup>. Although the gastrointestinal tract remains the most thoroughly investigated organ-microbiome interaction we hypothesized that the lung microbiome may also play a significant role in shaping the host immune responses in the

diabetic lung as the portal of entry of Mtb. To assess whether an altered microbiota in T2D contributes to increased susceptibility to TB disease, we compared the lung microbiome profiles of naïve and infected control and T2D mice (Figure 4-2A, B and S4-2). Actinobacteria, Firmicutes and Proteobacteria were the most abundant phyla in naïve-control and T2D lungs (Figure S4-2A). Mtb infection, however, led to a phylum-wide shift associated with a significant reduction of Actinobacteria in both groups and an increase in Bacteroidetes and Firmicutes (Figure S4-2A). The Shannon diversity index revealed an increase in overall lung community diversity in both control and T2D mice following the infection (Figure S4-2B). The significant expansion of families; Muribaculaceae, Lachnospiraceae and near complete absence of *Propionibacteriaceae* following infection was prominent (Figure 4-2A). Importantly, the increased TB burden in diabetic mice was detectable with Mycobacteriaceae levels being significantly increased in T2D mice (CON, 7.313±2.726% vs T2D, 34.17±6.851%, P=0.0219; Figure 4-2B). In addition to Mycobacteriaceae, pairwise comparison at family level uncovered 6 other differentially expressed families (DEFs) that were mutually increased in both TB and TB/T2D comorbid lungs compared to naïve control; Muribaculaceae, Lactobacillaceae, Lachnospiraceae, Ruminococcscrse, Erysipelotrichaceae and Akkermansiaceae (Figure S4-2C).

Whilst Firmicutes and Bacteroidetes were highly abundant in the guts of both naïve and infected mice, T2D mice harbored a significantly higher Firmicutes to Bacteroidetes ratio compared to control mice (Figure S4-2D). T2D gut microbiota was comprised of increased *Lachnospiraceae* and *Akkermansiaceae* abundance whilst higher levels of *Muribaculaceae* was noticeable in control mice (Figure S4-2E). Although not significant, overall gut microbiome diversity was lower in T2D mice and the reduction was further amplified following infection (Figure S4-2F, G). Interestingly, the highest number of DEFs in naïve T2D mice indicates marked alternations in the diabetic gut microbiota (Figure S4-2H-J).

Collectively, these findings suggest that the lung microbiota in T2D mice may not directly facilitate the increased susceptibility to aerosol *Mtb* infection.



#### Figure 4-2: Changes in lung microbiota and immune-related gene expression following Mtb infection.

Relative abundance of (A) total bacterial families and (B) family; mycobacteriaceae in lung tissues from control and T2D mice prior and 45 days post *Mtb* infection. (C) PCA plot and a heat map demonstrating unsupervised gene clustering of different clinical phenotypes. (D) The Venn diagram shows the DEGs in mice with TB and/ or T2D compared to naïve control mice (adjusted P < 0.05). Results are presented as (A) relative proportions and (B) pooled data means from 3-4 mice per group. \*P < 0.05 by unpaired 2-tailed Student's *t*-test (B). Data are means  $\pm$  SEM.

#### 4.3.3 Differential immune gene expression patterns following *Mtb* infection

To examine possible inherent immune defects that might be associated with TB/T2D comorbidity, we quantified expression levels of 548 immune-related transcripts in the lungs of control and T2D mice pre- and post-Mtb infection using NanoString nCounter. Principle component analysis (PCA) of the gene expression profiles separated samples into distinct clusters by both infection and disease status (Figure 4-2C). Unsupervised hierarchical clustering further confirmed distinct expression patterns amongst each clinical phenotype (Figure 4-2C). The highest variation in gene expression was seen between the naïve control and TB/T2D mice. Although, Mtb infection significantly altered gene expression patterns, there was less separation between TB (Mtb-infected control mice) and TB/T2D subgroups. A total of 389 differentially expressed genes (DEGs; adjusted P<0.05) were identified in the TB/T2D, TB and T2D compared to naïve control mice (Figure 4-2D and Table S4-1). 335 DEGs were identified in TB/T2D (vs control). Among those, 312 genes were mutually differentially regulated in TB/T2D and TB mice. These include genes previously found in blood and lung transcriptome profiles from TB patients and *Mtb*-infected mice, respectively<sup>627,628</sup>. In addition, 124 DEGs were differentially expressed between T2D and naïve control mice. Among those, 87 genes were shared between all 3 clinical phenotypes while 19 and 34 genes were uniquely differentially expressed only in T2D and TB/T2D, respectively. Interestingly, most of these DEGs are important for innate immune recognition and were downregulated in T2D and TB/T2D mice (i.e. Clec4a, Clec5a, Nod2, CD14). Collectively, the gene expression profiles identified here suggest that Mtb infection drives the majority of transcriptomic changes observed in infected mice, while T2D appears to cause the downregulation of genes that are crucial for *Mtb* recognition, control and for bridging innate and adaptive immune responses.

#### 4.3.4 ESX-1-containing BCG strains are safer than BCG in the context of T2D

The apparent defect in mycobacterial recognition in the lung in T2D prompted us to investigate the hypothesis that mucosal delivery of more immunogenic rBCG strains may provide a stronger immune response and, hence, better protection against TB in T2D. To this end we made use of experimental rBCG strains that have been shown to induce a more robust immune response<sup>48</sup>. We vaccinated both non-diabetic control and T2D mice with 3 different strains of BCG: parental BCG Pasteur (hereafter referred to as BCG), BCG::RD1 or BCG::RD1 ESAT-6  $\Delta$ 92-95 which both contain the extended RD1 locus from *Mtb* and secrete a full-length or a C-terminal truncated ESAT-6 protein, respectively. Each mouse received 5 × 10<sup>5</sup> CFUs of vaccine strain directly into the lung via the intratracheal (i.t.) route (**Figure 4-3A**). The superiority of mucosal BCG vaccination in mouse and NHP models<sup>266,629</sup> was re-confirmed in a pilot study (**Figure S4-3**). Throughout the 60 days vaccination period, animal wellbeing and body weight were monitored. At 30 days p.v. all vaccinated animals except BCG::RD1 ESAT-6  $\Delta$ 92-95 groups (both control and T2D) showed significant weight loss compared to unvaccinated animals. Interestingly, the most pronounced weight loss was seen in T2D mice that received conventional BCG (**Figure 4-3B, Table S4-2**). During the next 30 days, all groups started to regain lost weight. However, even at 60 days p.v. BCG-vaccinated T2D mice demonstrated significantly less body weight regain compared to all other groups (**Figure 4-3B**).

At 60 days p.v., mice from each vaccine group were sacrificed to assess bacterial clearance, lung tissue inflammation, cellular immune response and systemic cytokine/ chemokine profiles. The highest recovery of viable bacteria in lung and spleen tissues was observed from BCG-vaccinated T2D mice, suggesting that T2D may have impaired the clearance of BCG from the lung, which led to a widespread dissemination of bacilli to extra-pulmonary organs (Figure 4-3C). In contrast, control and T2D mice receiving either of the rBCG strains had significantly fewer bacteria in their lungs and spleen (Figure 4-3C). As expected, tissue inflammation was absent in non-diabetic unvaccinated lungs (Figure 4-3D). However, hematoxylin and eosin (H&E) staining revealed that unvaccinated T2D lungs were significantly more inflamed compared to unvaccinated control lungs confirming the chronic inflammatory nature of T2D (CON, 0.6270±0.1949% vs T2D, 5.113±1.038%, P=0.0004). There was no histological evidence of any organized lymphoid structures resembling inducible Bronchus Associated Lymphoid Tissues (iBALT) in unvaccinated T2D lungs when compared to vaccinated groups. The highest degree of lung tissue infiltration was observed in mice vaccinated with BCG and BCG::RD1 strains. Overall, the mildest histopathological changes were observed following mucosal vaccination with BCG::RD1 ESAT-6  $\Delta$ 92-95 (Figure 4-3D). Hence, BCG::RD1 ESAT-6 Δ92-95 had less impact on overall body weight (Figure 4-3B) and was more efficiently cleared from lung and spleen (Figure 4-3C) in both control and T2D mice.



Figure 4-3: ESX-1-containing BCG strains are safer than BCG in the context of T2D

Control and T2D C57BL/6 mice were vaccinated with  $5x10^5$  CFUs of BCG Pasteur, BCG::RD1 and BCG::RD1 ESAT6  $\Delta$ 92-95 via the i.t. route. (**B**) Body weight of the mice was measured during the p.v. period of 60 days. At 60 days p.v., mice were sacrificed and assessed for (**C**) clearance of vaccine strains in both lung and spleen and (**D**) immune cell infiltration in lung. Results are presented as (**B**) pooled data means  $\pm$  SEM, (**C**, **D**) individual data points and (**D**) representative images (Magnification, 25x; Scale bar, 500 µm) from 8-10 mice per group from two pooled independent experiments. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.001 by one-way ANOVA followed by Tukey's (**B**), Dunnett's multiple comparison test (**C**, **D**) and unpaired 2-tailed Student's *t*-test (**C**, **D**). Data are means  $\pm$  SEM

Overall serum cytokine and chemokine levels at day 60 p.v. were comparable among all vaccine groups in both control and T2D mice (Figure S4-4A). Increased fold changes in IL-10, Il-12p40 and KC (CXCL1) were seen across all the vaccinated groups in both control and T2D compared to unvaccinated mice (Figure S4-4B). The most noticeable fold decrease among all three vaccinated T2D groups was observed in MIP-1 $\alpha$  (CCL3); a cytokine with a known function of recruiting inflammatory cells<sup>630</sup>. Collectively, the absence of weight loss, the more efficient bacterial clearance and the reduced lung pathology indicate that genetically modified rBCG strains carrying ESX-1 are safer than conventional BCG in age-matched control and T2D mice.

# 4.3.5 ESX-1-containing BCG strains confer superior protection against aerosol *Mtb* challenge in both control and T2D mice

In the vaccination studies described above the ESX-1-containing BCG strains persisted for a shorter time and showed less lung pathology than the parental BCG. However, to assess if this enhanced safety also translated into superior protection, we infected mice with a very low aerosol dose of *Mtb* H37Rv (10-20 CFUs) 60 days after vaccination (Figure 4-4A). At 45 days after challenge, unvaccinated T2D mice had the highest number of detectable viable Mtb in lung and spleen tissues (Figure 4-4B). Parental BCG reduced lung bacterial loads in both nondiabetic and T2D mice by 5-10 -fold. This reduction has previously been shown in multiple studies in mice<sup>266</sup> and NHPs<sup>629</sup> following mucosal BCG vaccination. Intriguingly, when compared to unvaccinated and BCG-vaccinated groups, both rBCG strains significantly reduced lung *Mtb* burden not only in control, but also in T2D mice (Figure 4-4B). More strikingly, systemic spread of bacteria to the spleen was almost completely reversed by vaccination with ESX-1-containing BCG strains, with most mice showing sterile immunity in splenic tissues in both control and T2D mice. BCG-vaccinated mice presented with the greatest histopathological changes in both control and T2D lungs (Figure 4-4C). Consistent with bacterial loads, increased pathology was observed in unvaccinated and BCG vaccinated groups while rBCG groups showed only minor additional lung pathology post infection (p.i.) (Figure **4-4C**). The observed immunopathology among BCG-vaccinated animals, particularly in T2D may be attributed to the unresolved inflammatory lung microenvironment caused by mucosal BCG administration prior to aerosol *Mtb* infection (Figure 4-3D). Although systemic cytokine and chemokine levels were largely comparable between vaccine groups (Figure S4-4C), the majority of analytes were increased in T2D animals, among which IL-6, MIP-1β (CCL4) and TNF-α levels were significantly higher in rBCG-vaccinated T2D mice compared to BCG group

(Figure S4-4D). However, no significant difference was observed in control mice (Figure S4-4D). This suggest that the elevated systemic TNF- $\alpha$  levels in rBCG-vaccinated T2D animals were predominatly driven by the low-grade chronic inflmation associated with obesity in T2D<sup>631</sup>. Together these findings further substantiate the superiority of rBCG strains over conventional BCG in the context of T2D with superior protection and lack of systemic dissemination following aerosol *Mtb* infection.



Figure 4-4: *ESX-1-containing BCG strains confer a superior protection against aerosol Mtb infection in both control and T2D mice.* 

(A) 45 days post aerosol *Mtb* infection (10-20 CFUs), vaccinated and unvaccinated mice from both control and T2D groups were sacrificed and assessed for (B) viable bacteria in lung and spleen and (C) lung immunopathology. Results are presented as (B, C) individual data points and (C) representative images (Magnification, 25x; Scale bar, 500  $\mu$ m) from 8-10 mice per group from two pooled independent experiments. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\**P*<0.001 by one-way ANOVA followed by Dunnett's multiple comparison test (B, C) and unpaired 2-tailed Student's *t*-test (B, C). Data are means ± SEM.

#### 4.3.6 Superiority of ESX-1-containing BCG strains is associated with increased age

Given that near-sterile immunity against *Mtb* infection has not been reported previously with parenterally delivered (vaccination routes other than oral and/or mucosal) BCG::RD1 or BCG::RD1 ESAT-6  $\Delta$ 92-95<sup>48,56</sup>, we reasoned that our results could be related to the mucosal delivery of vaccines, the applied infectious dose and/or the advanced age of the mice used in challenge studies. Both non-diabetic control and T2D mice had been on a dietary intervention before being vaccinated, meaning that they were approximately 11 months old at the time of *Mtb* challenge (Figure 4-1A). To dissect the possible contribution of age, we vaccinated young (6 weeks) and old non-diabetic (36 weeks) mice (both fed with SD) with BCG, BCG::RD1 or BCG::RD1 ESAT-6  $\triangle$ 92-95 intratracheally and challenged them with a very-low dose of *Mtb* 60 days later (Figure 4-5A). At 45 days after Mtb infection, all vaccinated mice showed significantly reduced organ bacterial loads in lung tissues compared to unvaccinated mice (Figure 4-5B). However, aged mice that received BCG::RD1 showed a ~2-log reduction in lung CFU burden compared to unvaccinated mice, whereas young mice only displayed a ~1.3 -log reduction (Figure 4-5B). Similarly, compared to BCG-vaccinated mice, BCG::RD1 in aged mice had reduced lung CFU levels compared to young mice (~1.5 versus ~0.5 log reduction respectively). Age-related differences in lung pathology following infection were also noted with more inflammation seen in young compared to aged mice (Figure 4-5C). In this set of experiments, though we detected comparable CFU levels in both young and aged BCG::RD1 ESAT-6  $\triangle$ 92-95-vaccinated animals, lung pathology was significantly reduced in aged mice compared to young mice (Figure 4-5C). These results suggest that the superiority of ESX-1-containing BCG against TB is enhanced by advanced age and mucosal delivery of the vaccine.



Figure 4-5: Superiority of ESX-1-containing BCG strains is associated with advanced age.

(A) Young (6 weeks) and old (36+ weeks) C57BL/6 mice were vaccinated with  $5x10^5$  CFUs of BCG Pasteur, BCG::RD1 and BCG::RD1 ESAT-6  $\Delta$ 92-95 via i.t. route. At 60 days p.v., mice were infected with very-low dose (10-20 CFU) of *Mtb* H37Rv. At 45 days p.i., mice were sacrificed and assessed for (B) viable bacteria in lung and (C) lung pathology. Results are presented as (B, C) individual data points and (C) representative images (Magnification, 25x; Scale bar, 500 µm) from 6-7 mice per group. \**P*<0.05; \*\**P*<0.01 by unpaired 2-tailed Student's *t*-test (B, C). Data are means ± SEM.

# 4.3.7 ESX-1-containing BCG strains alter the immune cell composition and augment anti-mycobacterial function of APCs in the lung microenvironment

To investigate if the superiority of ESX-1-containing BCG strains was due to differential expansion or recruitment of immune cells in the respiratory tract, we performed comprehensive flow cytometry-based cellular phenotyping in both lung parenchyma (Figure 4-6A) and airways (Figure S4-5A) 60 days after i.t. vaccination. We analyzed 29 lymphoid and 8 myeloid cell subsets. In addition to total CD4<sup>+</sup> and CD8<sup>+</sup> T cells, we quantified central memory ( $T_{CM}$ ), effector memory ( $T_{EM}$ ) and resident memory ( $T_{RM}$ ) cells from each subset as they appear to play a key role in vaccine-induced immunity<sup>266,632</sup>. Cells were also stained with CD4 and CD8 MHC-peptide tetramers derived from the immunodominant *Mtb* antigens, ESAT-6 and TB10.4, respectively to measure mycobacteria-specific T cell responses. We also enumerated innate and innate-like cells, including polymorph-nuclear neutrophils (PMNs), dendritic cells (DCs), alveolar macrophages (AMs), interstitial macrophages (IMs) and mucosal-associated invariant T (MAIT) cells. We measured the fold change of each cell subset compared to the BCG-vaccinated group in both control and T2D mice (Figure 4-6A and S4-5A; raw data shown in Figure S4-5-S4-8).

Apart from the expected induction of ESAT-6-specific CD4<sup>+</sup> T cells, analysis of T cell subsets did not reveal consistent patterns across both rBCG vaccines and both groups of mice that might provide an explanation for the apparent protective efficacy and safety of these recombinant strains described above. B cells and MAIT cells showed a trend towards upregulation in all mice vaccinated with ESX-1-containing BCG strains. Strikingly, however, vaccination with these strains resulted in significantly increased numbers of DCs and macrophages (Figure 4-6A and S4-8E, F) in lungs from control and T2D mice. These results suggested that the superiority of mucosally administered ESX-1-proficient rBCG strains might be a consequence of altered innate APC function rather than T cell immunity. To further assess how rBCG vaccination may enhance the functionality of lung APCs, multiple *in vivo* and *ex vivo* functional/immunophenotyping assays were conducted (Figure 4-6B).

First, we tested if rBCG strains may overcome the defect in upregulation of co-stimulatory molecules in diabetes via preferential manipulation of co-stimulatory molecules. To this end, we used a previously reported LPS instillation model<sup>633</sup>. Twenty-four hours following i.t. LPS instillation, unvaccinated T2D mice displayed a significant downregulation of antigen recognition (i.e. CD209) and co-stimulatory molecules (i.e. CD40, CD80 and MHCII) on DCs

and macrophages from mediastinal lymph nodes (mLN) (Figure 4-6C, E) and lung parenchyma (Figure S4-9D) compared to unvaccinated control mice. However, vaccination with rBCG strains did not overcome this reduced expression compared to vaccination with BCG neither in T2D nor in control mice (Figure 4-6D, F and S4-9E). Similar results were obtained with *in vitro* infected bone marrow-derived dendritic cells (BMDCs) (Figure S4-9F, G).

Next, we investigated if the superior protection delivered by rBCG strains was due to increased T cell proliferation as a result of enhanced APC-T cell interaction. Using vaccine-stimulated APCs derived from control and T2D mice, we performed *in vitro* T cell proliferation assays with the well-established model system of ovalbumin specific CD4<sup>+</sup> (OT-II) and CD8<sup>+</sup> (OT-I) T cells. No significant differences in OVA-specific T cell proliferation between groups were observed (Figure S4-9H). In addition, the number of ESAT-6 and TB10.4-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the lymphatics prior to *Mtb* infection were not significantly different between non-diabetic and T2D mice (Figure S4-9I), suggesting that enhanced anti-TB immunity is likely also independent of APC-mediated T cell activation and proliferation.

Finally, we assessed if the ESX-1-containing BCG strains may impact on the ability of innate APCs to produce anti-mycobacterial compounds, such as reactive nitrogen species (RNS). Indeed, mice vaccinated with rBCG strains harbored significantly more inducible nitric oxide synthase<sup>+</sup> (iNOS<sup>+</sup>) cells including AMs and IMs (Figure 4-6A, G and S4-8G), suggesting an improved ability to generate oxygen and nitrogen species. The increased capacity of rBCG-vaccinated mice to generate nitric oxide (NO), was mirrored by increased nitrite concentrations in culture supernatants after infecting primary lung cells with *Mtb* for 24 hours (Figure 4-6H). In addition, *in vitro* rBCG infection also led to the generation of higher nitrite levels by bone marrow-derived macrophages (BMDMs) from control and T2D mice (Figure 84-9J).

Collectively, these findings indicate that mucosal vaccination with ESX-1-containing BCG strains not only generates more myeloid-derived immune cells in aged non-diabetic and T2D mice, but more importantly these cells appear to be endowed with an increased ability to mount anti-mycobacterial effector functions.





(A) Lung resident immune cell subsets at 60 days p.v. (B) Schematic representation of in vivo and in vitro APC functional assays performed. Costimulatory marker expression of mLN  $CD11c^+$  DCs from (C) unvaccinated and (D) vaccinated mice 24 hours after in vivo LPS instillation.  $CD11b^+$  F4/80<sup>+</sup> macrophages from (E) unvaccinated and (F) vaccinated mice were also assessed for costimulatory marker expression. (G) Total iNOS<sup>+</sup> lung cells 60 days p.v. and (H) nitrite production following 24 hours in vitro Mtb infection (MOI 1). Results are presented as a (A) heat map indicating log<sub>2</sub> fold changes relative to BCG Pasteur group and (C-H) pooled data means from (A, C, E) 8-10 and (D, F-H) 4-5 mice per group. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.001 by unpaired 2-tailed Student's t-test (C, E), two- way ANOVA (D, F) and one-way ANOVA followed by Dunnett's multiple comparison test (G, H). Abbreviations: ESAT6  $\Delta$ 92- 95; BCG::RD1 ESAT6  $\Delta$ 92-95, DN; double negative, T; T cells. Data are means ± SEM. See Figure S4-5-S4-9 for FACS gating strategies, raw data and representative FACS plots.

## 4.4 Discussion

It is now widely accepted that the intersection between TB and DM, poses a significant risk to the global TB eradication strategy<sup>8</sup>. Rationally designed new TB vaccine candidates are urgently required to meet the challenges of global TB eradication. Although there has been a significant progress over the last 2 decades to improve BCG by introducing *Mtb*-specific antigens and/or deletion of various genetic elements<sup>100</sup>, to our knowledge BCG or BCG-derived strains have not yet been evaluated as vaccines for TB in the context of T2D. Using our robust long-term diet-induced murine model of T2D, which embodies significant alimentary and polygenic factors involved in the development of clinical T2D, here we demonstrate increased susceptibility to TB in T2D following a very-low dose aerosol *Mtb* challenge that is thought to mimic natural TB infection in humans. Moreover, we provide evidence that mucosal delivery of rBCG strains that express RD1-encoded immunodominant T cell antigens<sup>48</sup> confer superior protection against pulmonary TB in T2D whilst maintaining heightened safety levels over conventional BCG. Our data point towards an important role of mucosal rBCG vaccination in inducing *Mtb*-specific lung- and airway-resident T cells and most importantly augmenting the anti-mycobacterial capacity of innate APCs in the lung.

Despite our expectation that parental BCG would have a better safety profile characterized by less bacterial persistence and dissemination, reduced tissue inflammation and immunopathology compared to the ESX-1-containing rBCG strains<sup>49</sup>, i.t. BCG vaccination resulted in significantly higher organ bacterial loads and more rapid body weight loss in T2D mice. We have previously demonstrated that 14 days after i.v. BCG infection, T2D mice had a higher bacterial burden in lungs, spleen and liver compared to control animals<sup>623</sup>. This was associated with impaired phagocytosis followed by defective BCG killing by alveolar macrophages<sup>518,623</sup>. The quantitative increase in T cell response and increased lung inflammation in BCG-vaccinated T2D mice may be a consequence of persistent organism/antigen due to inadequate clearance. Unlike conventional BCG, bacterial persistence and vaccine-induced total lymphocyte counts in T2D mice that received the rBCG strains were mostly comparable to the control group suggesting that these experimental vaccines act with similar efficacy in both conditions.

Live attenuated vaccines need to be adequately invasive and persistent to induce an effective expression of immunogenic antigens and to prime the immune system for subsequent exposures<sup>634</sup>. Using an NHP model of TB, Cadena and colleagues have recently reported that

local immune responses induced by primary Mtb infection were associated with a remarkable protection against reinfection with  $Mtb^{635}$ . This raises the important question if concomitant immunity should be the ultimate goal of live attenuated TB vaccines. Most importantly, these results further underscore that adding secreted Mtb antigens into BCG and mimicking the natural infection under attenuated conditions may be the best way to achieve vaccine-induced sterile immunity against TB infection.

RD1 is required for the full virulence of *Mtb* and encodes components of ESX-1; a type 7 secretion system and the absence of RD1 is vital for the attenuated virulence of the vaccine strain BCG<sup>49</sup>. However, the RD1 region is not the only virulence factor that differentiates *Mtb* from BCG<sup>636</sup>. Parenterally delivered BCG provides some protection against TB in animal models<sup>580</sup>. Although BCG confers greater protection against severe forms of miliary and meningeal TB in infants and children, incomplete protection against pulmonary TB in adolescents and adults has been one of its major failures<sup>101</sup>. In contrast, BCG::RD1 has shown improved vaccine efficacy against aerosol TB infection in murine and guinea pig models and the increased protection was attributed to ESAT-6 and CFP10 being crucial for mounting a maximal T cell response<sup>48</sup>. Despite the superior protection, BCG::RD1 and M. microti::RD1 knock-ins were associated with increased virulence in immuno-compromised animals and longer persistence in immuno-competent hosts following a very high dose of i.v. rBCG (10<sup>6</sup>-10<sup>7</sup> CFUs) immunization<sup>49</sup>, and hence were deemed unsuitable as human vaccines. Since then, numerous studies have provided compelling evidence that mimicking the natural route of infection is crucial for vaccine induced anti-TB immunity. Intranasal or intratracheal delivery of BCG provides increased protection against TB compared to traditional subcutaneous administration, and the enhanced protection is largely correlated with increased number of lung and airway  $T_{RM}^{266,632}$ .

Our current findings demonstrate that i.t. administration of ESX-1-containing rBCG induce equivalent protection against a challenge with virulent *Mtb* in both non-diabetic and T2D mice and the number of bacteria recovered was significantly lower than from BCG vaccinated mice. The superior protection was associated with reduced lung pathology with comparable serum cytokine/ chemokine levels across vaccine groups. Increased lymphocytes particularly *Mtb*specific activated T cells and higher frequencies of lung and airway  $T_{RM}$  may induce rapid recall response upon exposure to *Mtb*-containing aerosols, thus restricting bacterial replication and dissemination. Here in our long-term infection model, it is likely that the virulencemediated adverse effects of ESX-1 are outmatched by the immune-stimulatory effect in mucosal rBCG-vaccinated mice.

ESX-1-containing BCG vaccination induced a substantial influx of CD11c<sup>+</sup> DCs and CD11b<sup>+</sup> macrophages not only to non-diabetic control but also to T2D lungs. APCs are crucial for the activation and expansion of T cells. BCG::RD1 strongly enhances the ability of DCs to produce pro-inflammatory IL-1 $\beta$ , TNF- $\alpha$  and more importantly the capacity to expand IFN $\gamma^+$  T cells<sup>637</sup>. However, we could not detect any increase in DC co-stimulatory marker expression, induction of Mtb-specific T cells prior to Mtb infection nor increased T cell proliferation in vitro following vaccination/ stimulation with rBCG strains. In fact, a substantial reduction in almost every co-stimulatory surface marker compared to vaccination with BCG Pasteur was observed in primary lung and BMDCs following in vivo vaccination or in vitro infection, respectively, mirroring the active manipulation of APCs observed in natural *Mtb* infection<sup>638</sup>. Although we did not assess global cytokine or gene expression levels in innate cells, iNOS-expression and antimicrobial NO production were elevated in lung-resident AMs and IMs following rBCG vaccination. Conformably, rBCG's increased capacity to generate NO was also evident at the bone marrow level. This suggests that rBCG vaccination may have imprinted unique epigenetic changes into these innate APCs, which alters the rapidity and magnitude of the immune response to subsequent *Mtb* challenge. It has recently been shown that hematopoietic stem cells (HSC) derived from BCG vaccinated mice give rise to pulmonary and circulating monocytes and macrophages imprinted with Mtb-specific memory-like phenotype<sup>615</sup>. Although these results were obtained from a very high dose i.v. vaccination, and our study utilized a very-low dose mucosal vaccination, the impact on APCs in both situations could point towards an important role for trained immunity and need further investigation. Collectively, this emphasizes the pivotal role of RD1 in activation, recruitment and memory formation of both innate and *Mtb*-specific adaptive immune cells during TB infection in T2D mice.

Our results suggest that superior safety and efficacy seen for ESX-1-containing BCG against TB throughout this study, appears to be a consequence of advanced-age and mucosal delivery of the vaccine. The majority of mice currently being used in TB research are only 6 to 12-week-old, an age when animals are still undergoing physiological and developmental changes including in the immune system<sup>639</sup>. Although a definitive mechanism cannot be proposed, the increased safety and efficacy of ESX-1-strains in aged mice could be attributed to a number of age-related immune mechanisms including increase in age-associated B cells<sup>640</sup>, increased

exposure to innate immune training<sup>641</sup>, altered cytokine/chemokine milieu<sup>642</sup> and changes in microbiota<sup>643</sup>. Interestingly, our vaccination strategy in aged mice likely represents booster or revaccination in human adults rather than traditional neonatal BCG vaccination. This approach provides evidence of potential translational implications/applicability such as improving hyperglycemia in type 1 diabetes patients (possibly in T2D as well)<sup>644</sup> and enhancing trained immunity to exert non-specific protection against TB and/or TB-unrelated infections in adults<sup>643</sup>. Therefore, it is imperative to further investigate and unravel the impact of the age in the context of TB vaccine development.

Consistent with previous animal models<sup>376</sup> and T2D patients<sup>381</sup>, a markedly altered gut microbiota was observed in our diabetic mice. Interestingly, gut microbiota dysbiosis was associated with increased early susceptibility to *Mtb* infection and bacterial dissemination in mice<sup>365</sup>. How an altered intestinal microbiota in T2D influences anti-TB immunity is still unclear, but the concept of crosstalk between gut and lung microenvironments via the 'Gut-Lung Axis' is exciting and could be investigated in future studies<sup>352</sup>. Comparative immune gene expression analysis revealed that 16 out of 19 uniquely DEGs in T2D were to be downregulated and included genes associated with cell recruitment, pathogen recognition, cytokine production, and APC function. For an instance, Clec4e (Mincle) and Clec5a are important for the innate control and recognize carbohydrate-based mycobacterial cell wall components tetrahalose 6,6 dimycolate (TDM) and lipoarabinomannan (LAM)<sup>645</sup>. In addition to reduced lung CD14 transcripts, diminished MARCO expression in mLN macrophages and DCs was also detected (Figure S4-10); essential co-receptors for the TDM-induced immune response which have been associated with impaired innate recognition of *Mtb* in T2D mice<sup>515</sup>. Furthermore, CCL12, intracellular NOD2 and Lysosomal Cathepcin C, all of which have been implicated in immunity to  $Mtb^{628,646,647}$ , were also found to be significantly downregulated in T2D mice. Major transcriptomic changes were observed following Mtb infection. Among the 34 DEGs, 18 genes, the majority being innate immune response related; CXCL15, CCR1, IL18R and CD209 (DC-SIGN)<sup>56,648,649</sup>, were downregulated in TB/T2D suggesting that T2D associated innate defects are further exaggerated following Mtb infection. Similarly, recent integrative analysis of gene expression on South Indian TB/T2D comorbid patients revealed a unique gene signature also reflecting defective antigen processing/ presentation pathways<sup>530</sup>. Although *Mtb* strains in South India are often of Lineage 1, these similarities further strengthen the resemblance between comorbid human TB/T2D patients and our TB/T2D mice. Furthermore, the significant downregulation of these genes indicates that increased
susceptibility and disease exacerbation seen in T2D are likely associated with impaired early recognition and control of *Mtb* infection by innate APC subsets as evident by our functional APC assays.

In summary, our study provides a useful mouse model to investigate human TB/T2D comorbidity and to test TB vaccine efficacy against aerosol *Mtb* infection. Our findings accentuate that impaired innate recognition of *Mtb* in T2D within the lung exacerbates mycobacterial replication and TB disease. Moreover, mucosal vaccination with rBCG strains that express RD1-encoded *Mtb* antigens combines low virulence and improved protection against TB in aged non-diabetic and T2D mice. Together with our recent study showing superiority of ESX-1 containing strains in a systematic approach<sup>650</sup>, these findings warrant a reconsideration of 'virulent' BCG strains as effective vaccines in certain populations and a renewed focus on RD1-like BCG strains for TB vaccine development<sup>48,590</sup>. In addition, our study raises the important future questions of (i) understanding the impact of age on vaccine induced immunity to TB; and (ii) how new TB vaccine candidates interact with and 'train' innate APC subsets to augment anti-TB immune responses in the lung.

### 4.5 Supplementary Figures and Tables for Chapter 4



Figure S4-1: Lung immunopathology following aerosol Mtb infection.

Sub gross H&E stained lung histology images of control and T2D mice 45 days after *Mtb* challenge. (Magnification,10x; Scale bar, 1mm).



Figure S4-2: Changes in lung and faecal microbiota following aerosol Mtb infection.

Relative abundance of bacterial (A) phyla, (B) microbial alpha diversity in lung tissues and (C) a venn diagram representing DEFs in TB and/ or T2D compared to naïve control lungs; the horizontal bar graphs represent the fold changes of genus belongs to each family (adjusted P<0.05). Relative abundance of bacterial (D) phyla, (E) families and (F) microbial alpha diversity in faecal samples from control and T2D mice prior and 45 days after *Mtb* infection. (G) PCA of gut microbiota at the OTU level. Fold changes of differentially expressed families in (H) TB/T2D, (I) TB and (J) T2D compared to healthy controls (adjusted P<0.05). Results are presented as relative proportions (A, D, E), individual data points (B, F, G) and fold changes (G-J) from 4-5 mice. \*P<0.05 by one- way ANOVA followed by Tukey's multiple comparison test (C). Abbreviations: N/A; not available.



**Figure S4-3:** *Mucosal BCG vaccination confers improved protection against aerosol Mtb infection.* (A) C57BL/6 male mice were vaccinated with  $5x10^{5}$ CFU of BCG Pasteur, via s.c., i.m. and i.n. routes. 60 days p.v., mice were infected with ultra-low dose of *Mtb* H37Rv. Multiple p.i. readouts were obtained at 14, 28 and 45 days following infection. At 60 days p.v., mice were sacrificed and assessed for (B) clearance of vaccine strains in lung and spleen, (C) immune cell infiltration in lung and (D) serum cytokine/ chemokine levels. Infected mice were sacrificed at 4, 28 and 45 days p.i. and assessed for (E) viable bacteria in both lung and spleen tissues. Results are presented as (B, C, E) individual data points, (C) representative images (Magnification, 25x; Scale bar, 500 µm) and as (D) a heat map of log<sub>2</sub> concentrations from 5 mice per group. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.001 by one-way ANOVA followed by Dunnett's multiple comparison test (B, C, E). Data are means ± SEM.

lo<u>og\_</u>cconcc ((cogi/nh))

ΝN





Serum cytokine/ chemokine levels were measured at (A, B) 60 days p.v. and (C, D) 45 days p.i. Heat maps show  $\log_2$  concentration of each analyte (A, C). The horizontal bar graphs represent the fold difference in cytokine/ chemokine levels of each vaccine/ test group compared to (B) unvaccinated group or (D) BCG Pasteur group at 60 days p.v. and 45 days p.i., respectively. The grey bars indicate *P*<0.05.



**Figure S4-5:** *FACS-based phenotyping of BALF and lung-resident T cell populations.* Different immune cell populations from (A) BALF at 60 days p.v. and (B) lung cells at 45 days p.i. were phenotyped using FACS. Total lymphocyte (C), memory T cell (D) and tetramer-specific T cell (E) counts from lung and BALF (F-H) are shown in the bar graphs. Results are presented in a heat map indicating fold changes of different immune cell populations in each vaccination group relative to BCG Pasteur group (A, B) and pooled data means  $\pm$  SEM (C-H) from 8-10 mice per group from two pooled independent experiments. \**P* <0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.001 by two-way ANOVA (C, F) and one-way ANOVA followed by Dunnett's multiple comparison test (D, E, G-H). Abbreviations: DN; double negative. Data are means  $\pm$  SEM.



#### Figure S4-6: FACS-based phenotyping of lung-resident T cell populations at 60 days p.v.

(A) Different T cell populations were gated based on surface marker expression. Representative FACS plots of (B)  $CD3^+$   $CD4^+$  and (C)  $CD3^+$   $CD8^+$  T cell subsets; TEM, TCM, TRM and tetramer^+ T cells. Total lymphocyte (D), memory T cell (E) and tetramer-specific T cell (F) counts and % are shown in the bar graphs. Results are presented as representative images (A-C) and pooled data means ± SEM (D-F) from 8-10 mice per group from two pooled independent experiments. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.001 by two-way ANOVA (D) and one-way ANOVA followed by Dunnett's multiple comparison test (E, F). Data are means ± SEM.



Figure S4-7: FACS-based phenotyping of pro-inflammatory cytokine expressing lung-resident T cell populations at 60 days p.v.

Lung cells were stimulated with plate-bound CD3e (4 µg/ml) for 6 hours and (A) cytokine expressing T cell populations were gated based on surface and intracellular marker expression. Representative FACS plots of IFN $\gamma^+$  (B) CD3<sup>+</sup> CD4<sup>+</sup>, (C) CD3<sup>+</sup> CD8<sup>+</sup> and IL-17A<sup>+</sup> (D) CD3<sup>+</sup> CD4<sup>+</sup>, (E) CD3<sup>+</sup> CD8<sup>+</sup> T cell subsets. In addition, total lung cells were infected with *Mtb* (MOI 1) for 4 hours before staining for (F) IFN $\gamma^+$  total T and (G) T<sub>RM</sub> cells. IL-17A<sup>+</sup> (H) total T and (I) T<sub>RM</sub> cells were also quantified. Results are presented as representative images (A-E) and pooled data means ± SEM (F-I) from 8-10 mice per group from two pooled independent experiments. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.0001 by one- way ANOVA followed by Dunnett's multiple comparison test (F-I). Data are means ± SEM.



Figure S4-8: *FACS-based phenotyping of lung-resident innate and innate-like T cell populations and NO production at 60 days p.v.* 

Different (A) innate and (B) innate-like T (i.e., MAIT) cell populations were gated based on surface and intracellular marker expression. Representative FACS plots of (C) iNOS<sup>+</sup> macrophages (i.e., AMs, IMs) and (D) CD4<sup>+</sup>/CD8<sup>+</sup> MAIT cell subsets. Total innate (E), total macrophage (F), iNOS<sup>+</sup> macrophages (G) and MAIT cell (H) counts are shown in the bar graphs. Results are presented as representative images (A-D) and pooled data means  $\pm$  SEM (E-H) from 4-5 mice per group. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\**P*<0.001 by two-way ANOVA (E) and one-way ANOVA followed by Dunnett's multiple comparison test (F-H). Data are means  $\pm$  SEM.



#### Figure S4-9: Assessment of costimulatory marker expression and APC function.

(A) Lung and mLN DCs and macrophages were gated based on CD11c, CD11b and F4/80 expression. Representative FACS plots of mLN (B) macrophages and (C) DCs expressing costimulatory molecules. Costimulatory marker expression of (D) naïve and (E) vaccinated lung CD11c<sup>+</sup> DCs 24 hours after *in vivo* LPS instillation. (F) Uninfected and (G) BCG-infected (16 hours at MOI 10) BMDCs were also assed for costimulatory marker expression. (H) Representative FACS plots from *in vitro* OT-I & -II T cell proliferation using OVA peptide-pulsed BMDCs. (I) rBCG-induced ESAT6 and TB10.4-specific T cells in superficial cervical LNs. (J) BMDMs from control and T2D mice were infected with BCG strains *in vitro* (MOI 10, 24 hours) and nitrite levels in culture supernatants were measured using Griess reagent. Results are presented as (A-C, H) representative images and (D- G, I, J) pooled data means from 3-5 mice per group. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.0001 by unpaired 2-tailed Student's *t*-test (D, F, J), two-way ANOVA (E, G) and one-way ANOVA followed by Dunnett's multiple comparison test (I). Abbreviations: ESAT6  $\Delta$ 92-95; BCG::RD1 ESAT-6  $\Delta$ 92-95. Data are means  $\pm$  SEM. Supplementary to Figure 4-6.



Figure S4-10: MARCO expression in mLN cells.

Surface expression of MARCO in mLN macrophages and DCs were assessed using FACS in naïve control and T2D mice. Results are presented as pooled data means from 5 mice per group. \*P<0.05; \*\*P<0.01; by unpaired 2-tailed Student's *t*-test. Data are means ± SEM.

**Table S4-1:** *DEGs.* NanoString-derived uniquely DEGs in (A) TB/T2D, (B) TB and (C) T2D. Uniquely shared DEGs among (D) TB/T2D & TB, (E) TB/T2D & T2D, (F) TB & T2D and (G) TB/T2D & TB & T2D. The expression levels were relative to naïve control mice.

D



Х	logFC	logFC AveExpr		P.Value	adj.P.Val	
Ccrl1	-4.91668	5.793897	-5.75449	1.94E-04	0.000392	
Cfd	-4.48364	8.776554	-6.07727	1.27E-04	0.000271	
Vtn	-4.43499	5.022757	-5.06968	5.07E-04	0.000887	
Cd209g	-4.00496	4.151426	-5.14451	4.55E-04	0.000806	
Cd109	-3.72614	4.381312	-6.31069	9.36E-05	0.000212	
Hc	-1.76067	10.75735	-6.72116	4.49E-05	0.000111	
Cd55	-1.50274	10.40986	-6.61566	5.13E-05	0.000123	
Cxcl15	-1.45839	15.00146	-5.23187	3.46E-04	0.00064	
Ppbp	-1.17207	10.70742	-2.72314	2.12E-02	0.027677	
HIx	-1.10536	7.726488	-3.01787	1.25E-02	0.017173	
Tnfsf10	-0.91198	11.06427	-4.29245	1.47E-03	0.002368	
Icosl	-0.80353	9.320729	-3.10044	1.08E-02	0.015069	
Gata3	-0.80193	7.409326	-3.06648	1.15E-02	0.015932	
Csf2	-0.61695	6.254211	-3.14755	1.00E-02	0.013978	
115	-0.61084	5.913495	-2.77539	1.91E-02	0.025165	
C2	-0.58377	9.50784	-2.42839	3.48E-02	0.043782	
ll18r1	-0.36614	11.3917	-2.5159	2.99E-02	0.038158	
Ddx58	-0.36256	10.68529	-2.5083	3.03E-02	0.038554	
Ebi3	0.504288	7.864169	3.468589	5.76E-03	0.008291	
Ptpn6	0.527429	11.03422	2.949681	1.41E-02	0.019037	
ltgb2	0.615262	11.68452	2.789451	1.86E-02	0.024703	
Ptger4	0.645902	6.621214	4.074495	2.09E-03	0.003265	
Fcer1g	0.686962	10.43957	2.982153	1.33E-02	0.018068	
Tnfrsf1b	0.716377	9.515437	3.01332	1.26E-02	0.017255	
lfit2	0.733516	9.087622	3.630381	4.38E-03	0.006427	
Itgal	0.751909	10.69493	4.126073	1.92E-03	0.003033	
Cd80	0.778005	7.616402	2.71824	2.11E-02	0.027632	
Ccl3	0.816873	7.014141	2.500593	3.08E-02	0.038962	
lfi204	0.842145	8.169905	2.709473	2.14E-02	0.027893	
Batf	0.952591	7.887058	5.276948	3.24E-04	0.000617	
Fcgr2b	0.957621	11.32598	3.058248	1.17E-02	0.016111	
Hcst	1.229431	5.915199	2.854029	1.69E-02	0.022538	
Fcgr4	2.092422	10.8165	3.958223	2.74E-03	0.00419	
Camp	5.899863	5.091327	2.970377	1.42E-02	0.0192	

В

Х	logFC	AveExpr	t	P.Value	adj.P.Val
Jak3	-0.5916	8.83268	-3.07991	0.011238	0.017224
Nfkbiz	-0.4389	9.505665	-2.44498	0.033862	0.047048
ll23r	0.511616	6.532151	2.731756	0.020575	0.029794
Btla	0.668235	9.772656	3.307087	0.007596	0.01223
Ccl22	1.000409	9.066343	4.844935	0.000618	0.001297
Kira7	4.052138	4.076873	3.006511	0.013401	0.020124

С

Х	logFC	AveExpr	t	P.Value	adj.P.Val
Clec4e	-2.56197	9.302031	-5.77313	1.73E-04	0.002337
Clec5a	-1.67814	8.207164	-4.80904	6.53E-04	0.005791
Fcgr3	-1.49167	9.711962	-4.97904	5.04E-04	0.005039
Nod2	-1.45072	6.070113	-3.24988	8.62E-03	0.036254
ll18rap	-1.33604	7.722096	-7.00002	3.16E-05	0.000745
Ccl12	-1.28399	8.064503	-4.25301	1.57E-03	0.010277
Tgfbi	-1.23376	11.72178	-4.07578	2.09E-03	0.013072
Bcl3	-1.18518	7.556765	-6.53175	5.72E-05	0.001063
Cfp	-1.08821	9.791598	-6.86147	3.76E-05	0.000762
Csf2rb	-1.0249	8.922041	-4.57339	9.41E-04	0.007922
Bst1	-1.01164	10.30913	-3.89953	2.79E-03	0.015377
Cd14	-0.7286	9.50403	-2.99646	1.30E-02	0.047091
Ctsc	-0.69733	12.44361	-4.37534	1.29E-03	0.008974
C3	-0.53217	13.18642	-3.88079	2.88E-03	0.015669
Bst2	-0.41475	11.70093	-3.17322	9.56E-03	0.03893
Relb	-0.37389	9.829767	-3.06073	1.16E-02	0.043823
ll1rl1	0.574079	6.332428	3.654554	4.21E-03	0.020168
Lif	0.837737	5.885135	3.55512	4.97E-03	0.022412
II12a	0.942288	5.976288	3.9452	2.59E-03	0.014908

Х	logFC	AveExpr	t	P.Value	adj.P.Val
Cd163	-5.83975	5.230587	-5.34734	3.41E-04	6.37E-04
Hamp	-4.96244	3.996735	-3.58343	5.10E-03	7.41E-03
N0X4	-3.55/26	10.51386	-14.0984	4.48E-08	1.82E-06
Tir5	-3.40727	5 883379	-8.0420	9 17E-06	3.22E-05
Ccl11	-2.7234	6.572195	-6.02149	1.24E-04	2.67E-04
Ccl25	-2.61785	5.873261	-6.70628	4.60E-05	1.13E-04
Cd36	-2.61159	13.78051	-9.29377	2.46E-06	1.35E-05
Cdh5	-2.50637	11.44452	-8.25335	7.33E-06	2.66E-05
Tal1	-2.46577	8.039117	-10.3846	8.67E-07	8.13E-06
Mme Cfb	-2.42948	10.34687	-0.81794	3.97E-05	1.01E-04
Ptk2	-2.24872	10.133	-9.54755	1.91E-06	1.11E-05
Rorc	-2.15517	8.396448	-10.1547	1.07E-06	8.39E-06
Zeb1	-2.14636	10.33016	-8.79449	4.10E-06	1.88E-05
Tgfb2	-2.10263	8.286268	-7.16818	2.57E-05	7.04E-05
Icam2 Recom1	-2.08016	12.02483	-7.17825	2.54E-05	6.99E-05
Ski	-2.06266	9 585827	-0.77007	4.16E-06	1.00E-05 1.08E-05
ll17re	-2.02311	7.389656	-8.3995	6.25E-06	2.32E-05
Phipp1	-1.9882	9.739681	-10.3355	9.07E-07	8.13E-06
Cd97	-1.95998	11.84015	-5.73124	1.69E-04	3.50E-04
Nt5e	-1.94851	10.66389	-9.40444	2.20E-06	1.23E-05
FN1 Rdafrb	-1.91189	7 420229	-7.93047	1.05E-05	3.53E-05
ll6st	-1.88577	10.70767	-12.7367	1.21E-04	2.47E-06
Cmklr1	-1.87408	9.945274	-9.51627	1.97E-06	1.13E-05
Map4k2	-1.85949	9.326916	-10.539	7.53E-07	7.81E-06
Dpp4	-1.83847	10.21499	-7.8133	1.20E-05	3.85E-05
Ncam1	-1.8145	5.518538	-4.74418	7.24E-04	1.23E-03
Intst12	-1.81234	9.286729	-12.86/4	1.10E-07 1.35E-09	2.4/E-06
Cradd	-1.79647	7.248185	-3.91043	1.08E-05	3.60E-05
Phlpp2	-1.77877	8.424348	-9.58518	1.84E-06	1.08E-05
Арр	-1.77556	13.44317	-12.3098	1.69E-07	2.69E-06
Kit	-1.75729	8.229532	-7.05971	2.94E-05	7.70E-05
Cd9 Rdafb	-1.7444	13.58508	-9.72744	1.61E-06	1.04E-05
Abcb10	-1.68086	7.161522	-7.8437	1.40E-05	4.29E-05 3.77E-05
Cish	-1.65986	6.375158	-8.01822	9.52E-06	3.32E-05
Smad5	-1.65924	10.57925	-8.87856	3.76E-06	1.84E-05
Tgfb3	-1.64544	8.96922	-7.44536	1.84E-05	5.24E-05
Ctnnb1	-1.63834	13.28286	-12.5167	1.44E-07	2.65E-06
ADCD1a	-1.60454	9.073972	-6.84145	3.69E-00	0.88E-05
Runx1	-1.59667	10.47113	-10.8911	5.51E-07	6.64E-06
Tollip	-1.59657	10.62255	-12.4068	1.57E-07	2.69E-06
Csf1	-1.5965	9.7022	-7.92912	1.05E-05	3.53E-05
Ltb4r1	-1.59184	5.820161	-3.35898	7.24E-03	1.03E-02
C024a	-1.58902	11.39996	-10.2012	1.03E-06	8.39E-06
Mr1	-1.56073	9.299252	-8.80611	4.05E-06	1.88E-05
Abl1	-1.55925	8.5706	-10.0604	1.17E-06	8.70E-06
Mapk11	-1.54251	5.717609	-5.11875	4.09E-04	7.38E-04
Fcgrt	-1.51382	9.958011	-8.17231	8.01E-06	2.84E-05
Anr Tef4	-1.46015	9.850131	-7.53076	1.67E-05	4.86E-05
Cd164	-1.38954	12.10835	-9.63315	1.76E-06	1.08E-05
Cd99	-1.38795	9.523362	-5.09492	4.24E-04	7.62E-04
Bcl6	-1.37222	7.909813	-4.70164	7.71E-04	1.29E-03
Atg16I1	-1.33763	9.433942	-8.54693	5.33E-06	2.14E-05
Cd81 Notch2	-1.33548	12.75609	-9.45669	2.09E-06	1.18E-05
Mx1	-1.3282	8.867601	-7.06432	2.92E-05	7.70E-05
Ikbkap	-1.31001	8.553575	-9.13586	2.89E-06	1.53E-05
Bcap31	-1.30861	11.92329	-9.85632	1.42E-06	9.59E-06
Irf3	-1.30509	5.6421	-4.01408	2.31E-03	3.56E-03
Psmd7	-1.30208 -1.28814	11.09771	-1.002/9	1.13E-05	3.73E-05 8.39E-06
Cd34	-1.2819	11.72672	-6.55746	5.53E-05	1.31E-04
Abcf1	-1.27783	8.94144	-8.57585	5.17E-06	2.11E-05
Muc1	-1.27747	12.47041	-7.32376	2.13E-05	5.94E-05
Icam1	-1.27352	11.47777	-6.61012	5.17E-05	1.23E-04
Stat50 Traf4	-1.2/21/	10.10114	-8.97795	3.39E-06	1.71E-05 4 20E-05
Zbtb7b	-1.24447	8.608753	-7.62161	1.50E-05	4.48E-05
Adal	-1.24252	7.49603	-7.95736	1.02E-05	3.47E-05
Fadd	-1.23775	7.026383	-4.11074	1.97E-03	3.10E-03
Mapk14	-1.2214	10.34117	-8.92114	3.60E-06	1.78E-05
Nap4K4	-1.20219	9.771645 8.503002	-5.41693 -8.28507	2.04E-04	5.12E-04
Ikbkg	-1.19388	8.206877	-7.86116	1.14E-05	3.73E-05
Traf6	-1.1894	8.748378	-9.84852	1.43E-06	9.59E-06
C6	-1.18012	9.714371	-4.34149	1.36E-03	2.20E-03
Cd244	-1.1669	9.117984	-3.98792	2.41E-03	3.70E-03
lif3 Sro	-1.16025	7.943259	-5.50703	2.32E-04	4.57E-04
Ceacam1	-1.1458 -1.12880	9.80/804	-10.6693	0.70E-07	1.4/E-06 3.83E-04
Serping1	-1.11354	11.96175	-6.01081	1.14E-04	2.50E-04
Npc1	-1.11286	10.76022	-8.78859	4.13E-06	1.88E-05
ltga5	-1.10324	8.598003	-5.3604	2.86E-04	5.53E-04
Rae1	-1.09901	9.650894	-8.65757	4.74E-06	2.03E-05
Cul9	-1.09892	7 707860	-8.49224 -8.62030	5.65E-06	2.23E-05
Casp2	-1.09415	8.137646	-0.02938	+.00E-06 1.06E-06	2.00E-05 8.39E-06
Chuk	-1.06508	10.07991	-7.47076	1.79E-05	5.14E-05

Ube213         -1.04554         10.1837         -8.42951         6.05E-06           Ig72r         -1.03823         8.957463         -8.61087         4.98E-06           Corl2         -1.01212         9.029967         -5.73795         1.67E-04           I110h         -1.0056         11.54333         -8.7174         4.745E-06           Th61         0.99236         7.86477         -4.70365         7.68E-04           Tnfs173b         0.99236         7.86477         -4.70365         7.68E-04           Tnfs173b         0.99236         7.86477         -4.70365         7.68E-04           I118         0.99236         7.85477         -4.70365         7.68E-04           Pamc2         0.95344         1.61103         -8.51343         5.52E-06           Irak1         0.95124         8.782552         -7.05983         2.94E-05           Smad3         0.93202         7.97117         -4.98455         5.00E-04           Jak1         -0.93222         7.97117         -4.98435         5.00E-04           Jak1         -0.92029         12.0283         -7.12374         2.71E-05           C4a         0.87445         1.7106         5.12426         4.0850-0 <td< th=""><th>2276.05 2.066.05 2.066.05 2.066.05 1.296.03 2.356.04 8.2982-04 2.206.05 7.356.04 8.2982-04 8.786.04 7.356.04 9.3262.04 9.326.04 9.3260.0</th></td<>	2276.05 2.066.05 2.066.05 2.066.05 1.296.03 2.356.04 8.2982-04 2.206.05 7.356.04 8.2982-04 8.786.04 7.356.04 9.3262.04 9.326.04 9.3260.0
IgrZr         -1.03823         8.957463         8.61087         4.98E-06           Ccrl2         -1.01212         9.02967         -5.73795         1.67E-04           Ilf0rb         -1.0056         11.54333         -8.7174         4.48E-06           Tir3         -0.99034         7.72118         -6.0662         1.06E-04           Ilf0rb         -0.99034         7.72118         -6.0662         1.06E-04           Ilf8         -0.99034         7.72118         -6.0662         1.06E-04           Ilf8         -0.97889         7.93288         5.02516         4.70E-04           PsmC2         -0.95124         8.76252         -7.05983         2.94E-05           Smad3         0.93202         7.97717         4.94335         5.02E-04           Jak1         -0.92029         12.0283         -7.12374         2.71E-04           Jak1         -0.93326         10.18982         -3.88123         2.88E-03           Pdcd2         -0.83326         10.18982         -3.88123         2.88E-03           Pdcd2         -0.83326         10.18982         -3.88123         2.88E-03           Pdcd2         -0.83326         10.87647         -6.08982         1.02E-04 <td< th=""><th>2.06E-05 3.49E-04 1.29E-03 2.35E-04 2.20E-05 7.70E-05 8.29E-04 2.20E-05 7.70E-05 2.25E-04 4.34E-03 9.36E-04 6.33E-04 9.32E-04 4.34E-03 9.37E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 7.25E-04 7.2</th></td<>	2.06E-05 3.49E-04 1.29E-03 2.35E-04 2.20E-05 7.70E-05 8.29E-04 2.20E-05 7.70E-05 2.25E-04 4.34E-03 9.36E-04 6.33E-04 9.32E-04 4.34E-03 9.37E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-03 9.37E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 4.29E-05 7.23E-04 7.25E-04 7.2
Ceriz         1.01212         9.02967         5.73795         1.67E-04           H10rb         -1.0056         11.5433         8.7174         4.45E-06           H10rb         -0.09924         7.8477         4.70365         7.68E-04           Tinfs1         0.99924         7.8477         4.70365         7.68E-04           Tinfs1         0.99924         7.82477         4.70365         7.68E-04           U18         0.99934         17.21118         6.06662         1.08E-04           Psmc2         0.95394         11.61103         8.51343         5.52E-06           Smad3         0.93303         9.62236         6.73564         4.0E-05           Card9         0.93222         7.97717         4.94355         5.0E-04           Jak1         0.92029         12.0283         -7.12374         2.71E-05           C4a         0.87454         11.7106         -5.12426         4.0E-05           Pdotd2         0.83026         0.53232         4.93479         5.38E-04           Pmb7         0.83116         10.8767         -5.6331         4.2E-03           Tinf3         0.8042         8.11426         4.32E-03           Tafb         0.76341         1.087	3.49E-04 1.94E-05 2.25E-04 8.29E-04 2.20E-05 2.25E-04 8.29E-04 2.20E-05 1.10E-04 8.78E-04 7.35E-05 7.35E-04 2.51E-04 4.34E-03 9.92E-04 4.29E-05 9.92E-04 4.29E-05 9.92E-04 4.29E-05 9.725E-05 7.23E-04 2.48E-04 2.48E-04 2.48E-04 3.34E-03 3.48E-03 3.
HI0b         -1.0056         11.54333         -8.7174         4.45E-06           Tr3         -0.99236         7.85477         -7.0355         7.68E-04           Tr6f13b         -0.99024         7.21118         -0.0656         7.68E-04           Tr6f13b         -0.99024         7.21118         -0.0656         7.05E-04           Tr6f13b         -0.99024         7.21118         -0.0562         7.05983         1.6113           Psmc2         -0.95394         1.6113         8.51434         5.522-06         7.05983         2.94E-05           Smad3         -0.93303         9.62325         -6.7356         4.40E-05         5.7264           Card9         -0.93222         7.9717         -9.8435         5.00E-04         Jak1         -0.92029         12.0283         -7.1274         2.71E-05           Card         -0.92029         12.0283         -8.9176         5.38E-04         1.15E-04           Hi6         -0.80066         5.32024         -3.9374         6.03894         3.28E-05           Fad5         -0.81316         10.87967         -7.67631         1.41E-05           Mifat2         -0.79549         10.59477         6.09892         1.02E-04           Ifmar <t< th=""><th>1.94E-05 1.29E-03 2.35E-04 8.29E-04 8.29E-04 8.29E-04 8.29E-04 7.70E-05 1.10E-04 8.78E-04 7.38E-04 7.38E-04 4.34E-03 9.36E-04 6.33E-04 9.92E-04 6.33E-04 9.92E-03 9.75E-05 7.23E-04 2.82E-03 9.75E-05 7.23E-04 2.82E-03 9.75E-05 7.23E-04 2.82E-03 9.75E-05 7.23E-04 2.82E-03 9.75E-05 7.23E-04 2.48E-04 3.48E-03 6.33E-04 1.92E-02 1.92E-</th></t<>	1.94E-05 1.29E-03 2.35E-04 8.29E-04 8.29E-04 8.29E-04 8.29E-04 7.70E-05 1.10E-04 8.78E-04 7.38E-04 7.38E-04 4.34E-03 9.36E-04 6.33E-04 9.92E-04 6.33E-04 9.92E-03 9.75E-05 7.23E-04 2.82E-03 9.75E-05 7.23E-04 2.82E-03 9.75E-05 7.23E-04 2.82E-03 9.75E-05 7.23E-04 2.82E-03 9.75E-05 7.23E-04 2.48E-04 3.48E-03 6.33E-04 1.92E-02 1.92E-
Instant         Instant         Instant         Instant           TITA         0.99236         7.85477         4.70365         IOBE-04           TinfsT13         0.99234         7.85477         4.70365         IOBE-04           It18         0.99234         7.85477         4.70365         IOBE-04           Pamc2         0.99334         7.221118         6.06662         I.0EE-04           Pamc2         0.95394         116.1103         8.61343         5.22516           Smad3         0.93303         9.62295         6.73564         AUE-05           Smad3         0.93303         9.62295         6.73564         AUE-05           Card9         0.93222         7.97717         4.98435         6.00446         1.55E-04           Alk1         0.92029         1.20233         4.02585         3.35E-04           Pamb7         0.81316         11.08716         4.89139         5.7EE-04           Niac1         0.80428         8.184547         3.68394         4.32E-03           Pamb7         0.81316         11.08716         4.817644         1.77E-03           Or7949         0.571464         6.85653         3.38E-04           Niac2         0.73901         <	1.29E-03 2.35E-04 8.29E-04 2.20E-05 7.70E-05 7.70E-05 7.38E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.38E-03 9.36E-04 6.38E-03 9.2E-05 7.23E-05 7.23E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
113         -0.992.36         7.894.71         -4.10356         7.685.71           17167130         -0.99024         7.21118         -0.6662         1.05E-04           1118         -0.99034         7.21118         -0.0662         1.05E-04           1118         -0.99034         7.21118         -0.0662         1.05E-04           1118         -0.97889         7.33228         -5.02516         4.70E-04           1118         -0.95124         8.81235         5.22505         6.0564           111         -0.95124         8.782552         -7.05983         2.94E-05           Card         -0.93202         7.7777         -4.98435         5.00E-04           Jakt         -0.92029         12.0283         -7.1274         2.71E-05           Cda         -0.84754         11.106         5.12426         4.05E-04           Hfe         -0.8326         10.18982         -3.88123         2.88E-03           Podd2         -0.83066         5.35503         -3.987-04         1.81E-04           Niafc1         -0.81311         10.9716         4.9874         3.85E-04           Niafc1         -0.8136         9.17456         4.17644         1.77E-03           Niafc2	1.29E-03 2.35E-04 8.29E-04 2.20E-05 7.70E-05 1.10E-04 8.78E-04 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.33E-03 9.36E-04 6.38E-03 9.2E-04 4.29E-05 2.82E-05 2.82E-05 9.75E-05 7.23E-04 4.33E-04 3.38E-03 6.37E-04 1.92E-02
Thisf13b         -0.99034         7.721118         -6.06662         1.06E-04           Ili18         -0.99389         7.33248         -5.02516         -7.05983         -5.02516           Pamc2         -0.95394         11.61103         -8.15143         5.522-16           Smad3         -0.95394         11.61103         -8.15143         5.522-16           Smad3         -0.95394         11.61103         -8.15143         5.522-16           Card9         -0.93323         9.52295         -6.7356         4.40E-05           Card9         -0.93222         7.97717         -4.98435         5.00E-04           Oka4         -0.87454         11.7106         -5.12426         4.05E-04           H6         -0.83326         10.18902         -3.88123         2.886-03           Pado2         -0.83306         6.535023         -4.9477         5.38E-04           Pamb7         -0.81311         11.06716         -4.89139         5.76E-04           Niatc1         -0.81311         11.06716         -4.89139         5.76E-04           Niatc3         -0.79549         10.5977         7.69852         3.78E-05           Hinar         -0.79549         10.59777         6.98623         3.78E-	2.35E-04 8.29E-04 2.20E-05 7.70E-05 1.10E-04 8.78E-04 7.38E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.38E-03 9.92E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
II18         -0.97889         7.93328         -5.02516         4.70E-04           Psmc2         -0.9534         11.61103         -6.5134         5.52E-06           Trak1         -0.95124         8.782552         7.05983         2.94E-05           Smad3         -0.93303         9.623295         -6.7356         4.04E-05           Card9         -0.93222         7.7777         -4.98435         5.00E-04           Jak1         -0.92029         12.0283         -7.12374         2.71E-05           CAa         0.93222         10.44963         -6.00446         1.15E-04           Hifa         -0.93222         10.44963         -6.00446         1.15E-04           Hifa         -0.8672         10.48963         -6.00446         1.15E-04           Phot2         -0.83056         5.35503         -4.93175         3.98E-03           Phot2         -0.80042         8.184547         -6.86984         1.22E-04           Ifigr2         -0.79549         10.59477         6.08892         1.02E-04           Ifigr2         -0.79549         10.59477         6.08892         1.22E-04           Ifigr2         -0.79549         8.711426         -1.767631         1.41E-05	8.29E-04 2.20E-05 7.70E-05 1.10E-04 8.78E-04 7.38E-05 7.35E-04 4.34E-03 9.36E-04 6.33E-04 6.33E-04 9.36E-04 6.33E-04 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 4.248E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Pame2         -0.95394         11.61103         -6.5143         5.52E-06           Imak1         -0.95124         8.78252         -7.05934         4.40E-05           Simad3         -0.93030         9.623295         -6.7366         4.40E-05           Card9         -0.93020         7.02717         -4.98435         5.00E-04           Card9         -0.93222         7.09717         -4.98435         5.00E-04           Cida         -0.87454         11.7106         5.12426         4.05E-04           Hild         -0.8326         10.19892         -3.88123         2.88E-03           Pdcd2         -0.83096         6.539023         -4.93479         5.39E-04           Pdcd2         -0.83056         6.539023         -4.93477         5.39E-04           Pamb7         -0.81369         9.27477         6.09892         5.76E-04           Nitalc1         -0.8042         8.184577         7.69892         1.02E-04           Hinar1         -0.77013         10.87967         7.67631         1.41E-05           Cidpp         -0.74634         9.771464         6.85653         3.78E-06           Hinar1         -0.77051         10.21614         4.02992         2.5E-03	2.20E-05 7.70E-05 1.10E-04 8.78E-04 7.38E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 4.32E-03 9.36E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.37E-04 1.92E-02
Inski         -0.95124         8.782552         7.05983         2.94E-05           Smad3         -0.93303         9.623255         6.7356         4.06-05           Card9         -0.93222         7.7771         4.96435         5.00E-04           Jak1         -0.92022         7.7771         4.96435         5.00E-04           Jak1         -0.92029         12.0283         7.12274         2.71E-05           Marco         5.3222         7.97717         4.96435         6.00E-04           Jak1         -0.92029         12.0283         7.12274         2.71E-05           Marco         -0.83026         10.4963         -0.0446         1.15E-04           He         -0.83026         0.18962         -3.89129         5.88E-03           Pdd2         -0.83026         0.38560         -3.89149         5.38E-04           Traf5         -0.81311         11.08767         -6.98929         1.02E-04           Ing2         -0.7043         1.05477         6.08989         3.28E-05           Vilar2         -0.76949         8.11426         4.17644         1.72E-04           Imar2         -0.73901         10.73989         5.13775         3.97E-04           Imar2	7.70E-05 1.10E-04 8.78E-04 7.38E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.38E-03 9.36E-04 4.29E-04 2.26E-04 2.26E-04 2.26E-04 2.26E-04 2.26E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Smad3         -0.9303         9.62226         -6.7366         4.00E-05           Card9         -0.93222         7.97717         4.98435         5.00E-04           Jahl         -0.93222         7.97717         4.98435         5.00E-04           Jahl         -0.93222         7.12374         2.71E-05           C4a         -0.87454         11.7106         5.12426         4.05E-04           Hife         -0.8326         10.18982         3.88123         2.88E-03           Padc2         -0.83096         6.535023         -9.83479         5.38E-04           Padc2         -0.83156         9.32748         5.255355         3.56E-04           Pamb7         -0.81311         11.08716         4.89139         5.76E-04           Nifat2         -0.79694         8.115454         -1.76531         1.41E-05           Vifat3         -0.79694         8.115424         4.05E-04         1.7644           Nifat3         -0.73901         10.73969         5.11377         3.97E-04           Nifat3         -0.73656         10.6009         6.02165         1.32E-04           Hihad         -0.7361         1.2824         4.020272         2.5E-03           Nikb1         -0.736	1.10E-04 8.78E-04 7.38E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Card9         0.93222         7.97717         4.98435         5.00E-04           Jak1         -0.92029         12.0283         -7.12374         2.71E-05           CA         0.87454         11.7106         5.12264         0.65E-04           Iñ35         -0.86672         10.44963         6.00446         1.15E-04           Pdod2         -0.83096         6.535023         -4.93479         5.39E-04           Pdod2         -0.83096         6.535023         -4.93479         5.39E-04           Traf5         -0.81356         9.227485         -5.25555         3.35E-04           Psmb7         0.81311         11.08777         6.08994         1.022-04           Iñar1         -0.80042         8.184547         -5.83894         4.32E-03           Iñar1         -0.7013         10.87967         -7.67631         1.41E-05           Iñar1         -0.77649         15.1444         4.85655         3.78E-04           Iñar2         -0.73901         10.73989         5.13775         3.97E-04           Iñar2         -0.73901         10.73989         5.25069         3.8E-04           Iñar2         -0.73931         10.21614         4.02272         2.25E-03           I	8.78E-04 7.38E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.33E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Catus         0.50229         12.023         -7.1234         0.00209           C4a         0.92029         12.023         -7.1234         0.00209           C4a         0.92029         12.023         -7.1234         0.00209           C4a         0.87454         11.7106         5.12426         4.05E-04           Hfe         -0.83326         10.18982         -3.88123         2.88E-03           Pdcd2         -0.83396         6.539023         -9.93479         5.39E-04           Parto         -0.81311         11.08716         4.99139         5.76E-04           Pambr         -0.79549         10.59477         -6.09804         1.22E-04           Iffard         -0.79549         10.59477         -6.09824         1.22E-04           Iffard         -0.79549         10.59477         -6.09824         1.22E-04           Iffard         -0.79549         10.59477         -7.0531         1.41E-05           C1qbp         -0.74634         9.751464         -6.86553         3.78E-05           Iffard         -0.73901         10.73899         -6.13775         3.9FE-04           Vibids         -0.72677         8.93966         -5.25063         3.36E-04           Lip	7.38E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.33E-04 9.92E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Jakit         -0.92/02.9         12/02.8         -7.123/4         2.7/1E-05           CGa         0.98/02.9         12/02.83         -7.123/4         2.7/1E-05           Ifi35         -0.86672         10.44963         -6.00446         1.15E-04           Pidod2         -0.83026         0.18982         -8.8123         2.88E-03           Padod2         -0.83096         6.535023         4.93479         5.39E-04           Traf5         -0.81356         9.327485         -5.25355         3.3EE-04           Pmb70         -0.81311         11.08771         6.08924         -0.81349         -0.81349           Pmb70         -0.81311         10.87747         6.08924         -0.025-04           Ifing2         -0.75949         10.84747         -6.08924         -0.025-04           Ifing2         -0.76949         8.711446         +.82650         3.38E-04           Ifing2         -0.76949         8.711446         +.82650         3.38E-04           Ifing2         -0.76949         8.711446         +.82650         3.38E-04           Ifing2         -0.76949         8.71144         +.02507         3.28E-03           Ifing2         -0.76949         8.71144         +.02507 <t< th=""><th>7.35E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.33E-04 9.92E-04 6.33E-04 9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 2.48E-04 6.33E-04 2.48E-04 6.33E-04 2.48E-04 6.33E-04 2.48E-03 6.37E-04 1.92E-02</th></t<>	7.35E-05 7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.33E-04 9.92E-04 6.33E-04 9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 2.48E-04 6.33E-04 2.48E-04 6.33E-04 2.48E-04 6.33E-04 2.48E-03 6.37E-04 1.92E-02
C4a         -0.87454         11./106         -5.12426         4.05E-04           Hifs         -0.86427         10.44962         3.60446         1.15E-04           Hife         -0.83266         10.18982         3.88123         2.88E-03           Padcd2         -0.83096         6.553023         -4.93479         5.39E-04           Traf5         -0.81356         9.227485         -5.25355         3.35E-04           Psmb7         -0.81311         11.08716         -4.89139         5.76E-04           Ifing7         -0.79549         10.59477         -6.09894         1.02E-04           Ifing7         -0.79549         8.71426         -1.77631         1.41E-05           C1dpp         -0.74634         9.751464         -6.85653         3.78E-04           Psmb5         -0.73656         10.60099         -6.21561         1.372-04           Psmb5         -0.73651         10.21614         4.02272         2.5E-03           NRb1         -0.72677         8.98396         -5.20569         3.38E-04           Lp2         -0.67716         7.59205         -2.94105         1.43E-02           Lp3         -0.65981         2.01214         4.0562         2.1E-03           <	7.35E-04 2.51E-04 4.34E-03 9.36E-04 6.33E-04 6.33E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
IfI35         -0.86672         10.44963         -6.0446         1.15E-04           He         0.83326         10.1892         -8.8123         2.88E-03           Pidot2         -0.8306         6.535023         -4.94376         5.39E-04           Padot2         -0.81356         9.327485         -5.25355         3.56E-04           Pimb7         -0.81311         11.07716         -4.89139         5.7EE-04           Nitatc1         -0.80042         8.184547         -5.83894         4.32E-03           Ifma7         -0.9549         10.58477         6.09829         1.02E-04           Ifma7         -0.75949         8.711426         -1.7644         1.77E-03           Natato         -0.75949         8.711426         -4.767631         1.41E-05           Ords04         8.711446         +8.5653         3.78E-05           Ifma7         -0.75961         10.5098         -6.21576         3.78E-05           Ifma7         -0.75961         10.5098         -6.21576         3.36E-04           Tg/b1         -0.75651         10.20972         2.25E-03         3.36E-04           Lp2         -0.6776         7.59205         2.94105         1.43E-02           N/bh1	2.51E-04 4.34E-03 9.36E-04 6.33E-04 9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Hfe         -0.8326         10.18962         -3.88123         2.88E-03           Podot2         -0.83056         6.55023         4.93479         5.99E-04           Traf5         -0.81356         9.327485         5.25355         3.35E-04           Pamb7         -0.81311         11.08716         -4.89139         5.7EE-04           Jimar         -0.81311         11.08716         -4.89139         5.7EE-04           Jimar         -0.979549         10.59477         -6.09892         1.02E-04           Jimar         -0.77013         10.87967         -7.67631         1.41E-05           Jimar         -0.76949         8.71142         4.17644         1.77E-03           Jimar         -0.73656         10.60099         -6.02156         1.13E-04           Ikkib         -0.73651         6.00296         -6.2156         1.31E-04           Jikh1         -0.71631         10.21441         4.02972         2.55-03           Nikh1         -0.70133         10.21414         4.02972         2.55-03           Nikh1         -0.70133         10.21414         4.02972         2.55-03           Nikh1         -0.70133         10.21414         4.02972         2.55-03	4.34E-03 9.36E-04 6.33E-04 9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 4.637E-04 1.92E-02
Pidod2         -0.83096         6.535023         -4.94779         5.39E-04           Traf5         -0.81361         9.327485         5.255355         3.35E-04           Panb7         -0.81311         11.08716         -4.89139         5.7EE-04           Nitatc1         -0.80421         8.184547         -5.83894         4.32E-03           Mitatc1         -0.97549         10.59477         6.09829         1.02E-04           Mitat3         -0.77043         10.87967         7.67631         1.41E-05           Vitat3         -0.73901         10.73989         -5.13775         3.7EE-05           Mitat3         -0.73661         10.02972         2.25E-03           Mitbh         -0.72656         10.20972         2.25E-03           Mitbh         -0.72650         10.21414         4.02972         2.25E-03           Mitbh         -0.72650         10.21414         4.02972         2.25E-03           Mitbh         -0.72650         2.94105         1.43E-02           Lip2         -0.67716         7.52025         2.94105         1.43E-02           Display         -0.65281         10.2146         -3.05663         1.62124           Lip2         -0.67716         7.13099 </th <th>9.36E-04 6.33E-04 9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02</th>	9.36E-04 6.33E-04 9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Trafs         -0.81366         9.327485         -5.2355         3.35E-04           Pamb7         -0.81311         11.06716         -4.89139         5.7EE-04           Pamb7         -0.81311         11.06716         -4.89139         5.7EE-04           Jifng2         -0.78549         10.59477         -6.09922         1.02E-04           Imar1         -0.77013         10.87967         -7.67631         1.41E-05           Nikaci         -0.76994         8.711426         -4.17644         1.7E-03           C1dpb         -0.74634         9.751464         -8.6553         3.78E-04           Pamb5         -0.73651         1.41E-05         1.13E-04           Ikbib         -0.73651         0.6099         6.21561         1.13E-04           Ikbib         -0.73651         0.81699         6.21561         1.13E-04           Ikbib         -0.73631         0.81699         6.21561         1.3E-04           Ikbib         -0.7363         0.81614         4.02972         2.5E-03           Nikb1         -0.70251         0.61123         -5.24133         3.41E-04           Lip2         -0.67716         7.59205         -2.94105         1.43E-02           Lip2 <td< th=""><th>6.33E-04 9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02</th></td<>	6.33E-04 9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Symb7         0.81311         11.08716         4.89139         5.76E-04           Nitaic1         0.80042         8.184547         3.68394         4.32E-03           Infar2         0.90042         8.184547         3.68394         4.32E-03           Infar1         0.77613         10.87967         7.67631         1.41E-05           Nata1         0.77613         10.87967         7.67631         1.41E-05           C1dpp         0.74634         9.751464         6.85653         3.78E-06           Ifnar2         0.73901         10.73989         6.13775         3.97E-04           Nibb1         0.72667         8.83396         6.25069         3.36E-04           Nibb1         0.72677         8.83396         5.25069         3.34E-04           LCp2         0.67716         7.58205         2.94105         1.43E-02           LCp2         0.67716         7.58205         2.94105         1.43E-02           Parg         0.65581         2.01214         4.0706         1.87E-02           Parg         0.65581         10.21246         3.05663         3.2E-03           Parg         0.65267         1.02466         3.05663         3.2E-03           Parg	9.92E-04 6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Nitic1         -0.8042         8.184547         -3.63394         3.2E-03           Ifing7         -0.78549         10.59477         -0.69892         1.02E-04           Ifing7         -0.77913         10.87967         7.676331         1.41E-05           Nitac3         -0.769549         8.711426         -4.17644         1.77E-03           Or16994         8.711426         -4.85653         3.78E-05           Nitac3         -0.73656         10.60099         -6.02156         1.13E-04           Nitab         -0.73651         0.60099         -6.02156         1.13E-04           Nitab         -0.71631         10.27144         4.02972         2.25E-03           Nitb1         -0.7025         10.61123         -5.24133         3.41E-04           Lip2         -0.67716         7.59205         2.94105         1.43E-02           Lip2         -0.67716         7.59205         2.94105         1.43E-02           Lip2         -0.67716         7.59205         2.94105         1.43E-02           Lip2         -0.67716         7.102099         2.78601         1.87E-02           Ppang         -0.65286         7.102999         2.78601         1.87E-02           Fyn	6.38E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
-0.00042         0.104301         -0.30394         4.32E-03           Ifing72         -0.7549         10.5947         -7.67631         1.02E-04           Ifinar1         -0.77013         10.87967         -7.67631         1.41E-05           Otropes         8.711464         -6.85653         3.78E-05           Ifinar2         -0.73901         10.73989         5.113775         3.97E-04           Ikikbi         -0.72657         8.83396         -6.22565         1.38E-04           Ikikbi         -0.72657         8.83396         -5.25069         3.36E-04           Ikikbi         -0.72677         8.83396         -5.25069         3.34E-04           Lop2         -0.67716         7.58205         -2.94105         1.43E-02           Lop2         -0.67716         7.52025         -2.94105         1.43E-02           Parg         -0.65286         7.10299         -2.78601         1.87E-02           Fyn         -0.61867         10.22464         -3.05668         3.2E-03           Parg         -0.65284         10.22465         -3.05668         3.2E-03           Oto05         12.0452         -3.2E73         3.2E-04           Fyn         -0.61867         10.2246	2.26E-03 2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
umgr2         -0.79549         10.5947         -6.09892         1.02E-04           Iffar1         -0.77013         10.87967         -7.67631         1.41E-05           Niatos         -0.76994         8.711426         -4.17644         1.41E-05           C1dpp         -0.74634         9.751464         -6.86553         3.78E-05           Pamb5         -0.73656         10.60089         -6.02156         1.13E-04           Nkbb         -0.72677         8.98396         -5.25069         3.6E-04           Tgb1         -0.71635         10.21614         -4.02972         2.25E-03           Nikb1         -0.70255         10.61123         -5.24133         3.41E-04           Lop2         -0.67716         7.59205         -2.94105         1.43E-02           Pamg         -0.65286         7.10399         -2.78601         1.87E-02           Pamg         -0.65286         7.10399         -2.78601         1.87E-02           Fyn         -0.61867         10.22466         -3.80568         3.26E-03           Cxxit2         -0.60005         12.04552         -3.2573         8.27E-03           Iffih1         -0.52841         10.3344         -3.88517         2.86E-03	2.26E-04 4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Ifinar1         -0.77013         10.87967         -7.67631         1.41E-05           Nialo3         -0.7694         8.711426         4.17644         1.77E-03           Cidpp         -0.74634         9.751464         -6.85653         3.78E-05           Pimb5         -0.73901         10.73989         5.113775         3.97E-04           Ikikb         -0.73651         10.0298         -6.21565         1.132-04           Ikikb         -0.72677         8.983966         -5.25069         3.36E-04           Tglb1         -0.7025         10.61123         -5.24133         3.41E-04           Lop2         -0.67716         7.59205         -2.94105         1.43E-02           Parg         -0.65286         7.102946         -3.805668         3.26E-03           Parg         -0.6598         10.22461         -4.07625         1.41E-04           Lop2         -0.67716         7.58205         -2.94105         1.43E-02           Parg         -0.65286         7.103099         -2.78601         1.87E-02           Fyn         -0.61867         10.22466         -3.80568         3.26E-03           Cox12         -0.61055         10.22452         3.2572.03         1.87E-02      I	4.29E-05 2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Nifatc3         -0.76994         8.711426         -1.17644         1.77E-03           Clopb         -0.76994         8.711446         +8.56653         3.78E-06           Ifinar2         -0.73901         10.73898         -5.13775         3.97E-06           Pamb5         -0.73607         10.60089         -6.02156         1.13E-04           Ikibb         -0.72677         8.989966         -5.25069         3.56E-04           Tglb1         -0.71363         10.21614         -4.02972         2.25E-03           Nibb1         -0.07025         10.61124         5.24133         3.41E-04           Lop2         -0.67716         7.58205         2.24105         1.43E-02           Els1         -0.65282         7.130909         -2.78601         1.87E-02           Fyn         -0.61867         10.22466         -3.05628         3.26T-03           Fyn         -0.61867         10.22462         -3.2573         3.27E-02           Fyn         -0.61867         10.22466         -3.36568         3.26T-03           Film1         -0.52841         10.3344         3.88517         2.86E-03	2.82E-03 9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Ĉrigba         -0.74634         9.751464         -6.86563         3.78E-05           límaz         -0.73956         10.73989         -5.13775         3.97E-04           Penb5         -0.73656         10.60069         -6.02156         1.15E-04           Ikbikb         -0.72677         8.98396         -5.25069         3.36E-04           Urb1         -0.70153         10.21641         4.02972         2.25E-03           Nikb1         -0.70153         10.21461         4.02972         2.6116-03           Dep2         -0.67176         7.59205         -2.94105         1.43E-02           Dep3         -0.65286         7.130909         -2.8601         1.87E-02           Fyn         -0.61867         10.22466         -3.80568         3.2EF-03           Fyn         -0.61867         10.24452         -3.2573         3.27E-03           Ifh1         -0.52841         10.3344         -3.88517         2.86E-03	9.75E-05 7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Ima2         -0.73901         10.73898         -5.1377         3.97E-04           Psmb5         -0.7667         10.60089         -6.02165         1.13E-04           Ikikb         -0.72677         8.98996         -5.25069         1.3E-04           JRD1         -0.71363         10.21614         -4.02972         2.25E-03           JRD1         -0.7025         10.61123         5.24133         3.41E-04           Lop2         -0.67716         7.58205         2.94105         1.43E-02           Ebs1         -0.65285         7.10909         -2.78601         1.87E-02           Fyn         -0.61867         10.22466         -3.80568         3.2EF-03           Fyn         -0.61867         10.23466         -3.80568         3.2EF-03           Fyn         -0.61867         10.23466         -3.80568         3.2EF-03           Fyn         -0.61867         10.23466         -3.80578         3.2EF-03           Ifh1         -0.52841         10.3344         -3.88517         2.86E-03	7.23E-04 2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
Panb5         0.73656         10.60089         -6.02156         1.13E-04           Ikbkb         0.72677         8.98396         -5.25069         3.3E-04           Ikbkb         0.72677         8.98396         -5.25069         3.3E-04           Ikbkb         0.77033         12.1614         4.02972         2.25E-03           Nikb1         0.70025         10.61123         -5.24133         3.41E-04           Lip2         0.67716         7.59205         -2.94105         1.43E-02           Pparg         0.65286         7.10009         -2.78601         1.87E-02           Fyn         0.61867         10.22466         -3.80568         3.26E-03           Cxx12         0.60005         12.04552         -3.2573         3.27E-03           Ifh1         0.52841         10.3344         -3.88517         2.86E-03	2.48E-04 6.33E-04 3.48E-03 6.37E-04 1.92E-02
URBAD         0.73607         8.98996         -5.25069         3.56E-04           Tg/b1         -0.7367         8.98996         -5.25069         3.56E-04           Tg/b1         -0.7367         8.98996         -5.25069         3.56E-04           Mbb1         -0.7025         10.61124         -4.02772         2.25E-03           Mbb1         -0.7025         10.61124         -5.24133         3.41E-04           Lop2         -0.67716         7.59205         2.94105         1.43E-02           Eb1         -0.65286         7.10909         -2.78601         1.87E-02           Fyn         -0.61867         10.22466         -3.05626         3.26T-03           Cxh12         -0.60005         12.0452         -3.2573         3.27E-03           Ib1         -0.52841         10.3394         -3.88517         2.86E-03           Tab1         -0.52841         10.3394         -3.88517         2.86E-03	6.33E-04 3.48E-03 6.37E-04 1.92E-02
MRX         -0.7267/         8.995996         -5.20069         3.365-04           Tgb1         -0.71363         10.21614         4.02972         2.25E-03           NRb1         -0.70025         10.61123         -5.24133         3.41E-04           Lcp2         -0.67716         7.59205         -2.94105         1.43E-02           Elst         -0.6598         12.01291         4.0706         2.11E-03           Pparg         -0.65286         7.130909         2.78601         1.87E-02           Fyn         -0.61867         10.22466         -3.80568         3.26E-03           Cxcl12         -0.6005         12.04552         -3.2573         3.27E-03           Ifhi1         -0.52841         10.3394         -3.88517         2.86E-03	6.33E-04 3.48E-03 6.37E-04 1.92E-02
Igubi         -0.71363         10.21614         -4.02972         2.25E-03           Mikbi         -0.7025         10.61123         5.24133         3.41E-04           Lop2         -0.67716         7.58205         -2.94105         1.43E-02           Els1         -0.65286         1.2.01291         -4.0706         2.11E-03           Pparg         -0.65286         7.130909         -2.76801         1.87E-02           Fyn         -0.61867         10.22466         -3.05628         3.267-03           Cxh12         -0.61867         10.2346         -3.85617         2.87E-03           Hin1         -0.52841         10.3394         -3.8817         2.86E-03	3.48E-03 6.37E-04 1.92E-02
NINb1         -0.70025         10.61123         -5.24133         3.41E-04           Lop2         -0.67716         7.59205         -2.94105         1.43E-02           Elst         -0.6598         12.01291         -4.0706         2.11E-03           Pparg         -0.65286         7.130909         -2.76801         1.87E-02           Korld         -0.61867         10.22466         -3.80568         3.26E-03           Korld         -0.61867         10.3244         -3.80568         3.26E-03           Tubrt         -0.62841         10.3394         -3.88517         2.86E-03	6.37E-04 1.92E-02
Lop2         -0.67716         7.59205         -2.94105         1.43E-02           Ets1         -0.6598         12.01291         -4.0706         2.11E-03           Pparg         -0.65226         7.130909         -2.76801         1.87E-02           Fyn         -0.61867         10.22466         -3.80568         3.2EF-03           Cxh12         -0.60005         12.0452         -3.2573         3.27E-03           Hin1         -0.52841         10.3394         -3.8817         2.86E-03           Tubrt         -0.52841         10.3394         -3.4817         2.692-03	1.92E-02
Els1         -0.6598         12.01291         -4.0706         2.11E-03           Pparg         -0.65286         7.130909         -2.78601         1.87E-02           Fyn         -0.61867         10.22466         -3.80566         3.26E-03           Cxcl12         -0.60005         12.04552         -3.2573         8.27E-03           Iffin1         -0.52841         10.3394         -3.88517         2.86E-03	
Pparg         -0.65226         7.130909         -2.78601         1.87E-02           Fyn         -0.61867         10.22466         -3.80568         3.26E-03           Cxcl12         -0.60005         12.04552         -3.2573         8.27E-03           lifh1         -0.52841         10.3394         -3.88517         2.88E-03           Tefpet         -0.48827         10.63947         4.1814         4.95E-03	3.27E-03
Fpn         0.61867         10.2246         -3.8056         3.26E-03           Cxcl12         -0.61867         10.3246         -3.8056         3.26E-03           Ifih1         -0.52841         10.3394         -3.88517         2.86E-03           Tchr1         -0.52841         10.8917         41614         1.912-03	2 48F-02
vpr         -v.0.1007         10.2240b         -3.809b8         3.22E-U3           Cxcl12         -0.60005         12.04552         -3.2573         8.27E-03           Ifih1         -0.52841         10.3394         -3.88517         2.86E-03           Cmbr1         0.49827         10.69017         4.1614E         4.92E-03	4 905 00
OKCI12         -0.60005         12.04552         -3.2573         8.27E-03           Ifih1         -0.52841         10.3394         -3.88517         2.86E-03           Tofbr1         0.48597         10.69017         4.16115         1.97E 03	+.09E-03
Ifih1 -0.52841 10.3394 -3.88517 2.86E-03	1.18E-02
Tofbr1 0.49597 10.69017 4.16115 1.925.02	4.32E-03
-0.46367 10.06917 -4.10113 1.62E-03	2.88E-03
Cd82 -0.43311 11.41212 -3.53405 5.16F-03	7.47E-03
Jak20.41735_10.481573.88874_2.84E_03	4 31E-03
Irok2 0.39672 9.371394 3.69130 3.36E 03	2 01E 02
10.00072 0.071004 =2.00129 2.20E=02	2.910-02
Tyk2 -0.37896 7.363585 -2.70448 2.16E-02	2.81E-02
Traf3 -0.37145 9.061055 -2.91747 1.49E-02	2.00E-02
Bax -0.36285 9.124764 -2.5924 2.62E-02	3.35E-02
Casp8 -0.34709 9.455643 -2.9875 1.32E-02	1.80E-02
B2m 0.327547 16.77156 2.439651 3.42E-02	4.32E-02
Cd40 0.507258 8.796298 4.04401 2.20E-03	3 /1E-03
	4 425 02
Gpr163 0.514230 7.506417 5.666942 2.95E-03	4.43E-03
Pmi 0.533099 9.598346 3.50555 5.41E-03	7.81E-03
Cd226 0.607351 8.821145 2.72893 2.07E-02	2.72E-02
Tnfrsf14 0.646299 9.602774 5.048051 4.54E-04	8.06E-04
Tnfaip3 0.712337 9.194893 4.704073 7.68E-04	1.29E-03
Itgax 0.716284 10.40107 4.445215 1.15E-03	1.89E-03
Inf5 0.725937 9.615707 4.460555 1.12E-03	1.85E-03
Casp1 0.740448 0.685066 5.165304 3.815.04	7.005.04
U.749446 9.065066 5.165294 5.61E-04	7.00E-04
H2-DMa 0.792185 11.48776 4.434807 1.17E-03	1.91E-03
II2rg 0.805989 10.22644 5.441305 2.55E-04	4.98E-04
H2-K1 0.824058 13.25482 6.366382 7.10E-05	1.64E-04
Cd1d1 0.85762 9.333618 4.198885 1.71E-03	2.73E-03
Ccr7 0.884474 8.632395 3.580805 4.76E-03	6.97E-03
Cd74 0 942368 15 97871 6 05933 1 07E-04	2 37E-04
0.0502000 10.01011 0.000000 1.012-04	C 20E 04
Plau 0.959286 7.988323 5.235127 3.44E-04	6.39E-04
1.016759 8.867598 4.894651 5.73E-04	9.91E-04
Cd2 1.03716 10.35438 6.1112 9.98E-05	2.24E-04
Irf7 1.06113 7.817731 5.303852 3.11E-04	5.95E-04
Irf8 1.062595 9.416266 5.862479 1.40E-04	2.97E-04
Tagap 1.092394 8.449597 7.491482 1.75E-05	5.05F-05
Par5 1.099103 8.189481 3.773848 3.44E.02	5 12 -02
H2.Ag 1.112602 15 60461 6.404004 0.005 05	2.045.04
1.112092 13.00461 6.191291 8.96E-05	2.04E-04
1.131902 9.729/94 0.382851 0.95E-05	1.01E-04
LIITA 1.134082 9.924605 6.645749 4.94E-05	1.20E-04
H2-Ob 1.162105 9.04056 4.969222 5.12E-04	8.92E-04
Ly86 1.164256 10.23914 6.829868 3.91E-05	9.96E-05
Cd48 1.172211 10.32377 6.300813 7.75E-05	1.78E-04
Cd86 1.214307 8.457845 8.431575 6.03F-06	2.27E-05
Nfatc2 1 332132 7 440852 5 736607 1 67E 04	3 495-04
1.002102 1.770002 0.100001 1.0/E-04	5.44E 0E
Irom1 1 262204 11 75000 7 400474 4 005 05	J. 14E-05
Irgm1 1.363304 11.75223 7.466471 1.80E-05	2.23E-05
Irgm1         1.363304         11.75223         7.466471         1.80E-05           H2-Eb1         1.40381         13.73662         8.483326         5.71E-06	1.04E-04
Irgm1         1.363304         11.75223         7.466471         1.80E-05           H2-Eb1         1.40381         13.73662         8.483326         5.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05	1.47E-05
Irgm1         1.363304         11.75223         7.466471         1.80E-05           H2-Eb1         1.40381         13.73662         8.483326         5.71E-06           Cd83         1.431389         9.819006         6.784309         1.414E-05           H2-Ab1         1.44915         14.50637         9.18796         2.74E-06	7.52E-05
Irgm1         1.363304         11.75223         7.466471         1.80E-05           H2-Eb1         1.40381         13.73662         8.483326         5.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           H2Ab1         1.44915         14.50637         9.18796         2.74E-06           Cybb         1.490746         11.8185         7.104171         2.78E-05	
Igmin         1.363304         11.75223         7.466471         1.80E-05           M2-Eb1         1.40381         13.7626         8.463326         5.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           H2-Ab1         1.44915         14.50637         9.18796         2.74E-06           Cybb         1.44915         11.8185         7.104171         2.78E-05           Cd2         1.51951         8.51364         6.893072         3.61E-05	9.37E-05
Irgm1         1.363304         11.75223         7.468471         1.80E-05           M2Eb1         1.40381         13.76828         8.48326         6.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           M2Ab1         1.44915         14.50637         9.18796         2.74E-06           Cd23         1.44915         14.80674         1.18185         7.104171         2.78E-05           Cd22         1.51951         8.351364         6.83027         3.61E-05         2.65812         1.40472         3.755642         3.4927         3.61E-05	9.37E-05
Igmin         1.363304         11.75223         7.468471         1.80E-05           M2Eb1         1.40381         13.76828         8.483326         S.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           H2Ab1         1.44915         14.50037         9.18796         2.74E-06           Cd22         1.51951         8.53164         6.893027         3.61E-05           Tap1         1.555812         11.49142         8.735598         4.36E-06	9.37E-05 1.93E-05
Irgm1         1.363304         11.75223         7.468471         1.80E-05           M2Eb1         1.40381         13.73628         8.483326         S.71E-06           Cd83         1.413139         9.819006         6.784309         4.14E-05           M2Ab1         1.44915         14.50637         9.18796         2.74E-06           Cybb         1.44915         14.50637         9.18796         2.74E-06           Cd22         1.51951         8.351384         6.893027         3.61E-05           Cd22         1.51951         8.351384         8.36E-06         3.46E-06           Tmm2         1.563513         7.159262         4.849599         6.14E-04	9.37E-05 1.93E-05 1.05E-03
Igmin         1.363304         11.75223         7.468471         1.80E-05           VA2Eb1         1.40381         13.76828         8.483326         S.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           H2-Ab1         1.44915         14.50637         9.18796         2.74E-06           Cd22         1.51951         8.351364         6.893027         3.61E-05           Cd22         1.51951         8.351364         6.893027         3.61E-05           Tap1         1.555812         11.49142         8.735588         4.36E-06           Ikbke         1.628605         8.435683         8.767578         4.22E-06	9.37E-05 1.93E-05 1.05E-03 1.88E-05
Irgm1         1.363304         11.75223         7.468471         1.80E-05           M2Eb1         1.40381         13.7662         8.483326         S.71E-06           Cd83         1.413389         9.819006         6.784309         4.14E-05           Cd83         1.431389         9.819006         6.784309         4.14E-05           Cd83         1.44915         14.50637         9.18796         2.74E-06           Cd22         1.51951         8.351384         6.88027         3.61E-05           Cd22         1.51951         8.351384         6.88027         3.61E-05           Tem1         1.555812         1.414142         8.35684         3.68E-06           Tem2         1.653513         7.159262         4.849599         6.14E-04           1.628605         8.435683         8.7657578         4.22E-06           Cd5         1.642203         8.354566         6.13175         9.7E-05	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04
Igmin         1.363304         11.75223         7.468471         1.80E-05           VA2Eb1         1.40381         13.7662         8.48326         S.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           H2Ab1         1.44915         14.50637         9.18796         2.74E-06           Cd22         1.51951         8.35136         7.108796         2.74E-06           Cd22         1.51951         8.351364         6.893027         3.61E-05           Tap1         1.55812         11.49142         8.735598         4.36E-06           Tem2         1.55513         7.15222         2.445959         6.14E-04           Ikbke         16.266513         7.15222         2.495996         6.14E-04           Ikbke         16.28605         8.435688         8.767578         4.22E-06           Cc75         1.642203         8.354586         6.1175         9.71E-05           Pipn22         1.61169         8.98918         8.989799         3.42E-06	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05
Irgm1         1.363304         11.75223         7.468471         1.80E-05           VA2Eb1         1.40381         13.7562         8.483326         S.71E-06           Cd83         1.413389         9.819006         6.784309         4.14E-05           VA4015         1.450637         9.181906         6.784309         4.14E-05           Cd23         1.44915         14.50637         9.181906         6.784309         4.14E-05           Cd22         1.51951         8.351384         6.890327         3.61E-05           Cd22         1.51951         8.351384         6.890327         3.61E-05           Tem7         1.555812         1.149142         2.735598         4.42E-06           Cd22         1.555812         1.149142         2.735598         4.42E-06           Tem7         1.656351         7.159262         4.849599         6.14E-04           Ikbke         1.628605         8.435683         8.765778         4.22E-06           Cd75         1.624203         8.354586         6.13175         9.71E-05           Pipn22         1.661169         8.389818         8.969799         3.42E-06           Cd71         1.686515         7.36277         4.2227         4.252-07	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03
Igmin         1.363304         11.75223         7.468471         1.80E-05           VE2b1         1.40381         13.7662         8.48326         S.71E-06           C483         1.431389         9.819006         6.764309         4.14E-05           H2Ab1         1.44915         14.50637         9.18796         2.74E-06           C423         1.44915         14.50637         9.18796         2.74E-06           C422         1.51951         8.351384         6.83027         3.61E-06           Tem2         1.563513         7.159262         4.845996         6.14E-04           Ikkke         1.622605         8.435683         8.767767         4.22E-06           Cer5         1.642169         8.989418         8.969799         3.42E-06           Xer1         1.665965         7.236627         4.22E-06         Xer1           Ilia         1671093         6.71484         6.507769         3.42E-06	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-02
Irgm1         1.363304         11.75223         7.468471         1.80E-05           IV2Eb1         1.40381         13.7562         8.483326         S.71E-06           Cd83         1.413389         9.819006         6.784309         4.14E-05           Cybb         1.44195         14.50637         9.187906         2.74E-06           Cybb         1.44915         14.50637         9.187906         2.74E-06           Cybb         1.44915         14.50637         9.187906         3.61E-05           Cd22         1.51951         8.351364         6.893027         3.61E-05           Team         1.55512         1.41424         8.735594         3.44E-04           Team         1.563513         7.159262         4.849599         6.14E-04           Lebedos         3.835486         6.13175         9.71E-05           Pipn22         1.661169         8.989918         8.989799         3.42E-06           Car5         1.64194         8.989818         8.989799         3.42E-06           Car5         1.661169         8.989818         8.960479         4.90E-04           Kar1         1.671093         1.66248         4.960644         4.90E-04           Kibit <td< th=""><th>9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06</th></td<>	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06
Irgm1         1.363304         11.75223         7.468471         1.80E-05           IV2Eb1         1.00381         13.73628         2.483326         5.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           Cd83         1.431389         9.819006         6.784309         4.14E-05           Cd23         1.44915         14.50637         9.18796         2.74E-06           Cd22         1.51951         8.351364         6.893027         3.61E-05           Cd22         1.51951         8.351364         6.893027         3.61E-05           Trem2         1.563513         7.159262         4.84599         6.14E-04           Ikbke         1.622805         8.435683         8.767767         4.22E-06           Cd75         1.642203         8.345686         6.13175         7.1E-05           Pipn22         1.661169         8.98978         8.969799         3.42E-06           IIa         1.655865         7.236627         4.252643         1.57E-03           IIa         1.655965         7.236627         4.252643         0.60E-04           Statt         1.676128         9.825931         1.46E-06           Statt <td< th=""><th>9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05</th></td<>	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05
Irgm1         1.363304         11.75223         7.468471         1.80E-05           IV2Eb1         1.40381         13.7562         8.483326         S.71E-06           Cd83         1.413389         9.819006         6.784309         4.14E-05           Cybb         1.44195         14.5067         9.187906         2.74E-06           Cybb         1.44915         14.50637         9.187906         2.74E-06           Cybb         1.44915         14.50637         9.187906         3.76E-05           Cd22         1.51951         8.351384         6.893027         3.61E-05           Teap1         1.565513         7.159262         4.849599         6.14E-04           Teap1         1.565513         7.159262         4.849599         6.14E-04           Le30558         4.356868         6.13175         9.71E-05           Fibra         1.64203         8.354586         6.13175         9.71E-05           Pipn22         1.661169         8.989418         8.969799         3.42E-06           Carcf         1.671093         6.61483         4.506047         4.00E-04           UT         1.671093         6.61483         4.506047         4.0E-06           Lairi <td< th=""><th>9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05</th></td<>	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05
Irgm1         1.363304         11.75223         7.468471         1.80E-05           IV2Eb1         1.00381         13.73628         2.483326         5.71E-06           Cd83         1.413139         9.819006         6.784309         4.14E-05           T2AB1         1.44915         14.50637         9.18796         2.74E-06           Cd23         1.44915         14.50637         9.18796         2.74E-06           Cd22         1.51951         8.351364         6.893027         3.61E-05           Cd22         1.51951         8.351364         6.893027         3.61E-05           Trem2         1.563513         7.159262         4.849599         6.14E-04           Ikbke         1.628005         8.435683         8.76776         4.22E-06           Cd75         1.642203         8.354686         6.13175         7.1607           Pipn22         1.661198         8.89818         8.989799         3.42E-06           Xer1         1.665965         7.236627         4.256243         9.342E-06           Xer1         1.670928         1.85248         9.825931         1.46E-04           Statt         1.670617         8.441567         8.426851         6.06E-06	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05 4.53E-05
Irgm1         1.363304         11.75223         7.468471         1.80E-05           VA2B1         1.37628         2.483326         S.71E-06           C483         1.431389         9.819006         6.764309         4.14E-05           H2Ab1         1.44915         14.50637         9.18796         2.74E-06           C422         1.44915         14.50637         9.18796         2.74E-06           C422         1.51951         8.351364         6.83027         3.61E-06           Trem2         1.555812         11.49142         8.735598         4.36E-06           Ccc5         1.661503         8.435683         8.76776         4.22E-06           Kord         1.661969         8.989918         8.969799         3.42E-06           Xer1         1.667092         7.236627         4.22E403         1.57E-03           Ni1a         1.667109         6.61433         4.560447         9.60E-04           Start         1.677028         1.85248         9.25531         1.45E-06           Lair1         1.670028         1.8248         9.25631         1.45E-06           Lair1         1.676151         3.4927         7.59907         1.45E-06           Lair1         1.6761	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05 4.53E-05 2.39E-06
Irgam1         1.363304         11.75223         7.468471         1.80E-05           IV2Eb1         1.0031         13.73628         2.483326         5.71E-06           Cd83         1.4131389         9.819006         6.743309         4.14E-05           IL4915         14.50637         9.187906         6.748309         4.14E-05           Cd23         1.4490746         1.18185         7.104171         2.78E-05           Cd22         1.51951         8.351384         6.893027         3.61E-05           Cd22         1.51951         8.351384         6.893027         3.61E-05           Tem7         1.555812         1.149414         2.735594         3.42E-06           Cd75         1.642203         8.354686         6.13175         9.71E-05           Pipn22         1.661169         8.989818         8.999799         3.42E-06           Cd75         1.642203         8.354868         6.81375         9.60E-04           Ilia         1.671093         6.61483         4.560847         9.60E-04           Statt         1.670017         8.44157         8.265531         1.46E-06           Claif         1.76017         8.44157         8.265531         1.64E-06	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05 4.53E-05 2.39E-06 4.32E-06
Irigmi         1.363304         11.75223         7.468471         1.80E-05           VA2Eb1         1.40381         13.73628         2.483326         S.71E-06           C483         1.431389         9.819006         6.784309         4.14E-05           VA351         1.43015         9.819006         6.784309         4.14E-05           VA351         1.44915         14.50637         9.18796         2.74E-06           Cd22         1.51951         8.35136         6.7893027         3.61E-06           TemP2         1.556312         7.159262         4.849596         6.14E-04           Ikbke         1.622605         8.435683         8.767576         4.22E-06           Cref         1.66169         8.989918         8.969799         3.42E-06           Xcr1         1.665965         7.236627         4.252605         6.06E-06           Pip22         1.661169         8.989918         8.969799         3.42E-06           Xcr1         1.665965         7.236627         4.252605         6.06E-06           Lairi         1.67103         6.6148         4.560447         0.560-04           Stat1         1.67003         8.45578         4.25604         0.0E-07	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05 4.53E-05 2.39E-06 4.32E-06 7.70E-05
Irigmi         1.363304         11.75223         7.468471         1.80E-05           M2Eb1         1.00381         13.73628         8.483265         6.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           M2Ab1         1.44915         14.50637         9.18796         2.74E-06           Cd22         1.490746         11.8185         7.104171         2.78E-05           Cd22         1.51951         8.351364         6.89027         3.61E-05           Cd22         1.559512         11.49142         8.35684         3.68E-06           Trem2         1.653513         7.153262         4.849599         6.14E-04           Ikbke         1.62203         8.354566         6.13175         7.769778         4.22E-06           Cd5         1.682503         8.354636         6.13175         7.769778         4.22E-06           Cd5         1.681198         3.6819818         8.969799         3.42E-06           Cd7         1.6827023         8.354586         6.13175         9.60E-04           Statt         1.670017         4.41575         4.225831         1.46E-06           Clairi         1.7606178         4.1375         8.268561	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05 4.53E-05 2.39E-06 7.39E-06 7.70E-05 7.28E-03
Irgm1         1.363304         11.75223         7.468471         1.80E-05           IV2Eb1         1.00381         13.7562         8.48326         S.71E-06           C483         1.431389         9.819006         6.784309         4.14E-05           C483         1.44915         14.50637         9.18796         2.74E-06           C424         1.44915         14.50637         9.18796         2.74E-06           C422         1.51951         8.351364         6.893027         3.61E-05           C422         1.558512         1.149142         8.735598         4.36E-06           Trem2         1.563513         7.159262         4.849599         6.14E-04           Ikbke         1.622605         8.435683         8.767767         4.22E-06           C475         1.661169         8.989718         4.960793         3.42E-06           C471         1.661696         7.236627         4.25243         0.60E-04           C471         1.661696         1.84972         7.589907         1.46E-05           C481         1.76028         1.85248         8.25531         1.46E-06           C481         1.7515864         13.10E-07         1.8441557         1.92E-05 <t< th=""><th>9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 9.59E-06 2.27E-05 4.53E-05 2.39E-06 4.32E-06 7.70E-03 7.70E-03 8.39E-06</th></t<>	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 9.59E-06 2.27E-05 4.53E-05 2.39E-06 4.32E-06 7.70E-03 7.70E-03 8.39E-06
Irgm1         1.363304         11.75223         7.468471         1.80E-05           IV2Eb1         1.00381         13.7562         8.483326         S.71E-06           Cd83         1.431389         9.819006         6.784309         4.14E-05           IA490746         1.8165         7.104171         2.78E-05           Cd22         1.51951         8.351364         6.893027         3.61E-05           Cd22         1.51951         8.351364         6.893027         3.61E-05           Cd22         1.51951         8.351364         6.89999         6.14E-04           Ikbke         1.6263513         7.159262         4.849599         6.14E-04           Ikbke         1.626605         8.36583         8.767578         4.22E-06           Cdc5         1.64159         8.389818         8.969799         3.42E-06           Cdc5         1.651695         7.326647         4.90E-04         1.67003           ITa         1.671093         6.61433         5.7E-031           ITa         1.671093         6.61433         4.90E-04           ITa         1.671083         6.61433         5.7E-031           ITa         1.671083         6.61433         5.90E-031 <tr< th=""><td>9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05 4.53E-05 2.39E-06 4.32E-06 7.70E-05 7.28E-03 8.39E-06</td></tr<>	9.37E-05 1.93E-05 1.05E-03 1.88E-05 2.19E-04 1.71E-05 2.52E-03 1.60E-03 9.59E-06 2.27E-05 4.53E-05 2.39E-06 4.32E-06 7.70E-05 7.28E-03 8.39E-06

X	logFC	AveExpr	t	P.Value	adj.P.Val
Ltb	2.024975	10.15166	11.87561	2.40E-07	3.68E-06
Cxcl13	2.085936	10.12943	5.08588	4.42E-04	7.92E-04
C1qa	2.102955	11.0288	10.18912	1.04E-06	8.39E-06
Kirc1	2.110308	7.925728	8.609538	4.99E-06	2.06E-05
H2-DMb2	2.123993	10.66587	8.466252	5.81E-06	2.25E-05
Ccl2	2.1591	8.165961	7.265542	2.29E-05	6.33E-05
Tnf	2.212222	6.860394	9.062952	3.11E-06	1.63E-05
C1qb	2.216688	11.66919	9.654577	1.72E-06	1.07E-05
Slamf7	2.288686	9.336999	13.34686	7.68E-08	2.25E-06
Ccl19	2.289131	9.581225	9.040388	3.18E-06	1.65E-05
Tnfrsf4	2.295324	6.080497	5.677384	1.87E-04	3.82E-04
Cxcr6	2.332613	9.572401	9.608132	1.80E-06	1.08E-05
Cd3e	2.39937	7.917689	11.31849	3.81E-07	5.15E-06
Zap70	2.432178	6.07289	6.63579	5.00E-05	1.21E-04
Stat4	2.46038	5.457511	2.632784	2.53E-02	3.26E-02
Cd4	2.474694	7.129827	10.46179	8.08E-07	8.13E-06
Cfb	2.498099	11.05945	8.26431	7.24E-06	2.65E-05
Cd3d	2.591951	9.23357	12.47379	1.49E-07	2.65E-06
Ccl7	2.824216	5.375627	3.950348	2.81E-03	4.27E-03
Socs1	2.828019	8.3952	13.43025	7.23E-08	2.25E-06
Cxcr3	2.844969	7.881884	10.56746	7.34E-07	7.80E-06
Ccl8	3.336904	10.21126	4.911808	6.39E-04	1.09E-03
Lta	3.998811	5.665764	7.978816	1.26E-05	3.99E-05
Prf1	4.004479	4.601267	3.163734	1.03E-02	1.43E-02
Ccl4	4.290717	4.163104	4.229857	1.80E-03	2.86E-03
Cxcl1	4.381916	4.497573	3.852604	3.28E-03	4.90E-03
Fasl	5.117728	4.444861	6.812682	5.01E-05	1.21E-04
Cxcl10	5.230825	7.752893	7.943278	1.37E-05	4.26E-05
Ccl20	5.466432	8.040076	8.518878	7.43E-06	2.67E-05
II12rb2	5.699952	4.135883	5.722917	2.03E-04	4.04E-04
Cxcl9	6.021687	9.347117	8.002855	1.28E-05	4.02E-05
Ctla4	6.09763	4.376939	6.711725	5.67E-05	1.32E-04
Nos2	7.611209	4.485683	13.93191	8.07E-08	2.25E-06
Х	logFC	AveExpr	t	P.Value	adj.P.Val
lrf1	0.34734	12.36734	2.605635	0.025623	0.032838
Arhgdib	0.444522	12.61906	2.618554	0.025054	0.032295
Tmem173	0.604305	8.531677	3.699368	0.0039	0.005779
Ncf4	0.638884	9.716638	3.633238	0.004359	0.006417
Ccl9	0.753056	10.70354	3.018977	0.01249	0.017173
Fcgr1	0.918935	8.364236	3.31215	0.00753	0.01073
Ptafr	0.933079	8.666256	3.175635	0.009524	0.013358
Msr1	1.257339	7.848432	4.097946	0.002015	0.003153
Pou2f2	1.263359	8.433612	4.305457	0.00144	0.002327
X	logFC	AveExpr	t	P.Value	adj.P.Val
ll1b	-2.05568	9.404138	-4.01603	2.50E-03	0.014833
Itgam	-1.46864	9.228459	-4.93153	5.42E-04	0.005253
Lilrb3	-1.35596	9.726437	-4.39532	1.25E-03	0.008846
Lilrb4	-1.33097	11.08192	-5.28949	3.18E-04	0.003632
Tlr2	-1.06965	8.651977	-6.36334	7.13E-05	0.001223
Tyrobp	-0.97747	12.48119	-5.15411	3.88E-04	0.004325
Bid	-0.68189	8.114957	-4.90022	5.68E-04	0.005392
Tir8	-0.60807	9.20311	-3.60258	4.59E-03	0.021783
Cxcr4	-0.5212	10.41962	-3.86869	2.94E-03	0.015794

G

Х	logFC	AveExpr	t	P.Value	adj.P.Val	
Cxcr2	-4.65382	8.037401	-9.05027	4.20E-06	1.88E-05	
Lilra6	-3.80159	4.677362	-7.26363	2.85E-05	7.66E-05	
S100a9	-3.56542	13.67651	-6.05516	1.30E-04	2.78E-04	
S100a8	100a8 -3.42161		-6.09415	1.23E-04	2.65E-04	
H2-Q10	-3.40424	7.250077	-8.68879	4.62E-06	2.00E-05	
Csf3r	-2.9731	8.989675	-11.6993	2.77E-07	3.98E-06	
ll1r2	-2.91315	6.654162	-3.00974	1.33E-02	1.81E-02	
Fkbp5	-2.85114	7.528182	-13.8986	5.16E-08	1.92E-06	
Ccbp2	-2.65111	8.182028	-12.4957	1.46E-07	2.65E-06	
ll1rap	-2.48499	8.269295	-16.8577	7.61E-09	4.85E-07	
lfitm1	-2.17114	11.57651	-9.82554	1.46E-06	9.59E-0	
Notch1	-2.16181	10.03575	-10.8216	5.85E-07	6.87E-0	
ll6ra	-2 04558	9,90801	-10.0702	1.16E-06	8.70E-0	
Tirap	-1.94957	7.666206	-12.3265	1.67E-07	2.69E-0	
Tnfsf14	-1 92614	6 866887	-9.69116	1.66E-06	1.05E-0	
Trem1	-1.90339	7 203337	-5.3233	3.02E-04	5.81E-0	
Fas	-1.89326	9 492675	-16.372	1.02E-08	5.05E-0	
Tfrc	-1 67956	10 68772	-10.0808	1 15E_06	8 70E-0	
l tbr	-1 64025	9 912075	-14 2551	4 02E_08	1 79E-0	
Sall	-1.60/12	10 83678	-10 3087	9.02E-00	8 13E-0	
1133	1 6476	10.00070	7 06204	1.01E.05	2 47E 0	
Plaur	1 47061	0.090244	-1.90394	2 24E 06	1 70E 0	
1144	1 40000	0.241759	12 0270	1.0EE 07	2.47E 0	
Tofoin6	4 07470	5.341730	-12.9270	0.005.04	4.545.0	
Entrod1	1 22022	10 67005	10 2602	9.02E=04	0.12E-0	
Stat6 1 2120		11 26696 0 92202		0./9E-0/	0.13E-0	
ll4ro	410 -1.2129		-9.03393	1.430-00	9.09E=0	
Rolo	-1.1202	0.007004	-9.00/13	1.0/E-00	1.05E-0	
Maalut	-1.1104	9.907334	-10.3439	9.00E-07	0.13E-0	
марк і	-1.09900	11.21437	-10.3557	0.90E-07	0.13E-0	
Тил	-1.0754	10.93044	-0.24140	0.30E-05	1.92E-04	
00	-1.07469	9.007191	-9.63000	1.45E-00	9.59E-0	
CCIZ	-1.0/43/	10.87556	-5.161/1	3.84E-04	7.01E-0	
TDK I	-1.07105	9.494836	-8.83592	3.93E-06	1.88E-0	
51813	-1.04583	11.97806	-10.5776	7.28E-07	7.80E-0	
li i Sra i	-1.0424	10.95647	-0.0002	5.20E-00	2.14E-0	
Ingri	-1.01991	12.3801	-7.32333	2.13E-05	5.94E-0	
CCID	-0.99566	10.03294	-4.4613	1.12E-03	1.85E-0	
XDD1	-0.99431	12.09257	-7.64349	1.46E-05	4.40E-0	
муавв	-0.97233	9.750979	-7.61464	1.51E-05	4.49E-0	
Mapkapk2	-0.87221	10.49697	-7.78136	1.25E-05	3.97E-0	
ll1/ra	-0.84336	9.720526	-5.05899	4.47E-04	7.98E-0	
Cdkn1a	-0.79895	10.37203	-4.48034	1.09E-03	1.81E-0	
Nfkbia	-0.77806	9.670031	-5.43855	2.56E-04	4.98E-0	
Irak4	-0.64242	9.765335	-5.67982	1.81E-04	3.72E-0	
Socs3	-0.60442	7.935988	-2.67145	2.29E-02	2.95E-0	
Irak2	-0.57233	9.511018	-4.42533	1.19E-03	1.94E-0	
Nfil3	-0.5319	8.218637	-2.43192	3.46E-02	4.36E-0	
Litaf	-0.52046	9.452201	-3.24346	8.47E-03	1.20E-0	
Cd44	-0.3692	11.62889	-3.22887	8.69E-03	1.22E-0	
Ptpn2	-0.34298	9.494064	-2.83316	1.72E-02	2.30E-0	
Kird1	0.872615	9.014726	5.73991	1.66E-04	3.49E-0	
Cd79b	1.267693	10.62186	5.207313	3.58E-04	6.61E-0	
Cd19	1.385825	9.51958	5.610348	2.00E-04	4.00E-0	
ll10ra	1.518172	9.291146	7.014299	3.11E-05	8.10E-0	
ll27ra	1.645926	7.827148	11.1884	4.25E-07	5.42E-0	
Kiek 1	1,716847	8.206127	9.987552	1.25E-06	9.16E-0	

Cx3cr1 Spn Meda1	1.859032 1.871736	9.138877	6 664971	4 925 05	4 405 04
Spn Me/191	1.871736			4.02E=03	1.16E-04
Me/a1		9.805675	6.587835	5.32E-05	1.26E-04
1113401	1.899748	10.42428	7.674448	1.41E-05	4.29E-05
Cxcr5	1.96396	6.956453	5.70719	1.74E-04	3.60E-04
Blnk	2.012256	8.734815	10.31178	9.27E-07	8.13E-06
Cd247	2.237478	7.343304	13.61231	6.33E-08	2.17E-06
Irf4	2.304286	7.486999	8.233181	7.49E-06	2.67E-05
Map4k1	2.363585	6.817696	7.595282	1.54E-05	4.53E-05
Xcl1	2.56379	6.436491	11.83205	2.48E-07	3.69E-06
Lck	2.794015	9.180827	17.04718	6.81E-09	4.85E-07
Cd7	2.801852	5.703376	3.281125	8.43E-03	1.19E-02
Ccr6	3.083038	7.57243	8.455147	5.88E-06	2.26E-05
Ccl5	3.083654	11.43321	11.14856	4.40E-07	5.45E-06
Batf3	3.341828	4.868334	3.390306	7.02E-03	1.01E-02
Cr2	3.397526	5.357114	4.843292	7.07E-04	1.20E-03
Cd27	3.962251	5.907232	5.763371	1.92E-04	3.90E-04
Pdcd1lg2	4.045769	5.744608	5.747755	1.96E-04	3.94E-04
lcos	4.135562	7.413427	11.27878	3.94E-07	5.17E-06
Gzmb	4.165238	4.558646	7.574945	2.03E-05	5.72E-05
Runx3	4.176481	5.96409	5.366765	3.32E-04	6.30E-04
Cd5	4.220944	6.855934	18.13368	3.67E-09	3.27E-07
Slamf1	4.240191	5.650152	6.012368	1.38E-04	2.93E-04
ll12b	4.257746	5.801917	5.71458	2.05E-04	4.07E-04
Cd8a	4.32044	6.177333	9.018251	4.03E-06	1.88E-05
Eomes	5.54845	4.824742	25.90398	9.96E-11	1.48E-08
Cd69	5.808745	4.895092	16.56004	9.09E-09	5.05E-07
Pdcd1	6.320008	4.718494	23.70957	2.45E-10	2.73E-08
ll21r	6.57325	4.97036	13.30068	1.18E-07	2.47E-06
Cd6	6.981051	5.09553	29.61289	2.55E-11	5.68E-09
Sh2d1a	6.987842	5.461152	29.88572	2.32E-11	5.68E-09

**Table S4-2:** *Weight differences among vaccinated mice during the 60 day p.v. period.* Weight comparison of mice at (A) 30 days and (B) 60 days p.v. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001; \*\*\*P < 0.001 by one-way ANOVA followed by Tukey's multiple comparison test. Abbreviations: Unvac; unvaccinated,  $\Delta$ ESAT6; BCG::RD1 ESAT6  $\Delta$ 92-95, ns; not significant.

Α										
		Unvac	BCG	BCG::RD1	ΔESAT6	Unvac	BCG	BCG::RD1	ΔESAT6	CON
	Unvac		****	**	ns	ns	****	****	ns	T2D
	BCG	****		ns	*	**	***	ns	ns	
	BCG::RD1	**	ns		ns	*	****	ns	ns	
	ΔESAT6	ns	*	ns		ns	****	***	ns	
	Unvac	ns	**	*	ns		****	****	ns	
	BCG	****	***	****	****	****		****	****	
	BCG::RD1	****	ns	ns	***	****	****		ns	
	∆ESAT6	ns	ns	ns	ns	ns	****	ns		

В

	Unvac	BCG	BCG::RD1	∆ESAT6	Unvac	BCG	BCG::RD1	ΔESAT6
Unvac		ns	ns	ns	ns	***	ns	ns
BCG	ns		ns	ns	ns	ns	ns	ns
BCG::RD1	ns	ns		ns	ns	ns	ns	ns
ΔESAT6	ns	ns	ns		ns	**	ns	ns
Unvac	ns	ns	ns	ns		**	ns	ns
BCG	***	ns	ns	**	**		ns	ns
BCG::RD1	ns	ns	ns	ns	ns	ns		ns
ΔESAT6	ns	ns	ns	ns	ns	ns	ns	

## **Chapter 5**

## Disparate effects of metformin therapy on Mycobacterium tuberculosis infection in diabetic and non-diabetic mice

This chapter contains the manuscript titled 'Disparate effect of metformin therapy on tuberculosis in diabetic and non-diabetic mice' which has been submitted as a Brief Report and is currently under review in *Antimicrobial Agents and Chemotherapy*.

#### 5.1 Abstract

Comorbid type 2 diabetes (T2D), poses a great challenge to the global control of tuberculosis (TB). The immunomodulatory properties of the anti-diabetic drug Metformin (MET) have received attention in improving disease outcome in TB/T2D comorbid patients. Here we tested the efficacy of MET in diabetic and non-diabetic mice infected with a very low dose of *Mycobacterium tuberculosis* (*Mtb*). In contrast to diabetic mice, infected non-diabetic mice that received the same therapeutic concentration of MET presented with significantly higher disease burden. This warrants further studies to investigate the disparate efficacy of the MET against TB in diabetic and non-diabetic individuals.

#### 5.2 Background

Tuberculosis (TB) remains one of the most prevalent and deadliest infectious diseases globally with an estimated annual mortality of 1.5 million people and nearly 1.7 billion latently infected people worldwide <sup>8</sup>. Infections with drug-susceptible *Mycobacterium tuberculosis (Mtb)*, the causative agent of TB, can be successfully treated with long-term antibiotic therapy. However, rapid emergence of drug resistant strains and increasing incidence of comorbid conditions among *Mtb*-infected individuals, such as diabetes mellitus (DM), pose a great challenge to TB eradication<sup>651</sup>. It is estimated that 463 million people are currently living with diabetes and the numbers are expected to escalate to 700 million by 2045<sup>436</sup>. Diabetics have a threefold increased risk of developing active TB<sup>18</sup> and demonstrate a strong association with multi-drug-resistance (MDR) TB<sup>652</sup>. Therefore, robust anti-TB treatments and co-management strategies are essential for optimal treatment outcomes in comorbid patients.

Poor treatment adherence and clinical complications, such as empyema and extensive cavitation, combined with continuous exposure to conventional anti-TB monotherapy often lead to drug tolerance and resistance<sup>653</sup>. Several new and existing repurposed anti-TB drugs such as delamanid, bedaquiline and rifapentine are currently being developed or evaluated<sup>89</sup>. In addition, there has been an increased interest in non-antimicrobial host-directed therapies (HDT) which often target and improve host immune responses with the potential to shorten treatment duration and to improve the treatment output against all forms of TB<sup>89</sup>.

Metformin (MET; 1,1-dimethylbiguanide) is a widely prescribed AMP-activated protein kinase (AMPK)-activating anti-diabetic drug. Interestingly, multiple retrospective cohort studies have shown that MET therapy was also linked with reduced TB risk among diabetic

patients<sup>654,655</sup>, suggesting the potential use of MET as an adjunctive anti-TB therapeutic agent, particularly in the context of T2D. In line with this hypothesis, Singhal and colleagues demonstrated that MET inhibits intracellular growth of *Mtb* in C57BL/6 mice and improves the efficacy of the conventional first-line anti-TB drug; isoniazid (INH)<sup>655</sup>. However, in a more recent mouse study, MET failed to enhance the potency of current anti-TB treatment regimen in BALB/c mice<sup>656</sup>, implying the need for further animal studies to resolve the discrepancies between these findings, in particular in the context of diabetes.

Using a murine model of T2D<sup>543</sup>, we evaluated the protective efficacy of MET alone and in combination with INH against TB in both T2D and non-diabetic mice. Our findings indicate that MET restricts bacterial persistence in the lungs following a very-low dose *Mtb* infection in T2D mice. Intriguingly, non-diabetic mice that received the same MET dose exhibited a significant escalation in viable lung bacilli and increased lung immunopathology. These findings point towards disparate effects of MET in *Mtb*-infected diabetic and non-diabetic hosts, and warrant further pre-clinical and clinical studies to re-evaluate the protective effect of MET in non-diabetic healthy individuals.

#### 5.3 Results

#### 5.3.1 Murine model of T2D

Following 30 weeks of diet intervention (Figure 5-1A), mice fed with EDD presented with a significantly increased body weight (Figure 5-1B) and elevated fasting blood glucose levels (Figure 5-1C) compared to control mice that received SD. Impaired glucose tolerance (Figure 5-1D) was observed in EDD-fed mice as reflected by area under the curve (AUC). Confirmed T2D and non-diabetic control mice were subjected to downstream experimental procedures.



Figure 5-1: Diet induced model of murine T2D, Mtb infection and treatments.

(A) Six to eight weeks old C57BL/6 male mice were fed with EDD and SD (control mice) for 30 weeks to induce murine T2D. Following dietary intervention mice were assessed for (B) body weight, (C) fasting blood glucose levels and (D) glucose tolerance. (D) Glucose tolerance test was performed by measuring glucose concentrations at 15, 30, 60, and 120 mins post i.p. glucose administration (2 g/kg) and calculating AUC. (A) Diabetic and non-diabetic control mice were infected with very-low aerosol dose (10-20 CFUs) of *Mtb* H37Rv. Seven days p.i., MET (500 mg/kg), INH (10 mg/kg) and combination of MET + INH were administered in drinking water. Results are presented as individual data points (B-D) and pooled data means  $\pm$  SEM (D) from two pooled independent experiments. Statistical analysis: \*p < 0.05; \*\*p < 0.01; \*\*\*\*p < 0.001 by Student's *t*-test. Abbreviations: EDD; energy dense diet, SD; standard diet, i.p.; intraperitoneal, AUC; area under the curve.

#### 5.3.2 Divergent effects of metformin

To investigate whether metformin could restrict the growth of *Mtb* and enhance the efficacy of the first-line anti-TB drug INH, we infected non-diabetic control and T2D mice with a very-low aerosol dose of *Mtb* H37Rv (10-20 CFUs) which closely mimics the human TB. Mice which showed both CFUs on 7H11 agar plates and *Mtb* bacilli by Ziehl-Neelsen (ZN) stain were included. Due to the absence of viable CFUs on agar plates 5 mice were excluded from analyses despite confirmed presence of *Mtb* using ZN staining and increased lung inflammation at 45 days p.i. (Figure S5-1 and S5-2). Prior to the commencement of treatments, some mice were sacrificed to determine initial lung bacterial loads. CFU recovered from lung tissue 7 days p.i. revealed that both control and T2D mice carried a similar bacterial burden prior to treatment (Figure 5-2A). Forty-five days post aerosol challenge, the remaining mice that were on treatments were euthanized to assess *Mtb* burden in lung, lung tissue inflammation and

systemic cytokine/ chemokine profiles. Although not statistically significant, higher numbers of viable bacteria (T2D,  $log_{10} 4.581\pm0.4366$  vs CON,  $log_{10} 4.163\pm0.1670$ ; mean±SEM, p=0.3436) (Figure 5-2B) and substantially increased lung inflammation (T2D, 24.52±2.363 % vs CON, 17.74±2.343 %; mean±SEM, p=0.0645) (Figure 5-2C, D and Figure S5-1) indicated increased susceptibility to aerosol *Mtb* infection in T2D mice.

Administration of 10 mg/kg INH sterilized *Mtb* infection in almost all animals except in two T2D mice (**Figure 5-2B**), thus combination therapy with 500 mg/kg MET did not further enhance the efficacy of INH in our experimental settings (**Figure 5-2B**). Both INH and MET+INH treatments were also accompanied by significantly lower lung immunopathology (**Figure 5-2C, D** and **Figure S5-1**). Compared to untreated T2D mice, T2D mice that received MET had a significantly lower overall bacterial burden (~1.5-log reduction; **Figure 5-2B**). Strikingly, in contrast to a previous report<sup>655</sup>, non-diabetic control mice treated with MET showed a statistically significant increase in *Mtb* burden (**Figure 5-2B**), marginally increased lung pathology (UNT,  $17.74\pm2.343$  % vs MET,  $21.70\pm2.894$ ; mean $\pm$ SEM, *p*=0.3251) (**Figure 5-2C, D**) and unchanged pro-inflammatory IL-12p40 levels reflecting an ongoing inflammation (**Figure 5-2E**). This disparate efficacy of MET in non-diabetic and T2D mice resulted in a ~2-log difference in lung *Mtb* burden. Overall, inflammatory serum cytokine/ chemokine levels were comparable between control and T2D mice (**Figure S5-3A**) and all treatments downregulated the majority of analytes (**Figure S5-3B**).



Figure 5-2: Divergent effects of MET on T2D and non-diabetic mice.

Seven days p.i., group of mice were sacrificed and assessed for (A) lung bacterial loads. Forty-five days following infection, treated and untreated mice from both control and T2D groups were assessed for (B) viable bacteria in lung, (C, D) lung inflammation and (E) serum IL-12p40 levels. Results are presented as individual data points (A, B, D, E) and representative images (C) from two pooled independent experiments (n=7-10 mice per group). Statistical analysis: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001 by Student's *t*-test (A, B, D) and One-way ANOVA followed by Dunnett's multiple comparison test (B, D, E) from 7-10 mice per group from two pooled independent experiments. Abbreviations: CON; control, T2D; type 2 diabetes, CFU; colony forming units, p.i.; post infection, UNT; untreated, MET; metformin, INH; Isoniazid.

Collectively, our findings suggest that MET treatment improves the outcome of *Mtb* infection in T2D mice whilst having a detrimental impact on non-diabetic control animals in a very-low dose aerosol *Mtb* infection setting. In addition, 45 days after *Mtb* infection, a commonly used time-point in experimental murine TB, MET had little to no impact on enhancing the therapeutic efficacy of INH in both non-diabetic and T2D mice.

#### 5.4 Discussion

Immunomodulatory properties of MET have recently received considerable attention as an adjunctive anti-TB therapy. Cumulative evidence from recent meta-analysis<sup>657</sup> suggest that MET prescription is associated with a significantly lower incidence of active TB among TB/DM comorbid patients. The improved health outcome of TB/DM patients using MET included reduced mortality, fewer pulmonary cavities and rapid culture conversion. In addition, Singhal and colleagues showed that MET-treated DM patients had reduced LTBI prevalence and enhanced Mtb specific T cell responses as determined by T-SPOT assay<sup>655</sup>. In contrast, using QuantiFERON-TB Gold In-tube, Magee, et al. could not find an association between LTBI incidence in DM patients taking MET compared to non-MET DM patients<sup>658</sup>. Collectively, these reteroscpetive studies indicate that MET has the propensity to improve the overall treatment outcome when used with existing anti-TB regimens in TB/DM comorbid patients. Conformably, our T2D mice that were treated with a therapeutic concentration of MET displayed a significant improvement in disease outcome as reflected by reduced viable lung Mtb CFUs, lung immunopathology and serum IL-12p40 levels, compared to untreated T2D mice exposed to very-low dose aerosol Mtb. However, MET was not able to enhance the sterilizing effect of INH in infected animals as shown previously<sup>655</sup>. These discrepant findings may be due to differences in analysis time points (35 vs 45 days p.i.) and/or a reduced INH dose (5 vs 10 mg/kg) used previously<sup>655</sup>.

More strikingly, in contrast to the T2D mice, aerogenic *Mtb* infection resulted in augmented lung bacillary loads and increased lung immunopathology in non-diabetic mice that received MET. The increased bacterial persistence indicates diminished anti-TB immunity in MET-treated control mice. In a recent study, *ex vivo* MET administration diminished the production of number of *Mtb* lysate-induced pro-inflammatory cytokines in healthy human peripheral blood mononuclear cells (PBMCs)<sup>659</sup>. In addition, gene expression analysis also revealed that *in vivo* administration of MET downregulated the type 1 interferon pathway in healthy PBMCs<sup>659</sup>.

The potential role of MET as an adjunctive therapy for TB is exciting. However, current evidences for MET-induced anti-TB protection have come mostly from from retrospectively evaluated studies involving TB/DM comorbid patients. A number of studies have provided evidence for MET-induced reduction in pro-inflammtory threshold via possible inhibition of mammalian target of rapamycin (mTOR)<sup>660,661</sup> and/or perturbed gut microbiota<sup>366,662,663</sup>. While

this immunomodulatory effect of MET can be favourable in certain high-risk population such as diabetics with chronic inflammation, the excessive host anti-inflammatory responses can exert negative influence on the early control of *Mtb* and bacterial dissemination as observed in our non-diabetic mice. Further pre-clinical studies are therefore warranted to decipher the potentially disparate effects of MET in diabetic and non-diabetic hosts before it gains entry into clinical trials as an adjunctive anti-TB drug. In future, our long-term T2D mouse model will enable us to investigate the efficacy and optimum therapeutic concentration of MET against TB at various stages of the development of diabetes.

### 5.5 Supplementary Figures and Tables for Chapter 5



**Figure S5-1:** *Lung tissue inflammation.* Representative Hematoxylin and Eosin stained lung tissue sections from each mouse. Mice that have been excluded are highlighted in red (refer to **Figure S5-2**). Abbreviations: CON; control, T2D; type 2 diabetes, UNT; untreated, MET; metformin, INH; Isoniazid.



**Figure S5-2:** *Excluded samples.* Total of 5 mice were excluded from (A) CON MET, (B) T2D untreated and (C) T2D MET groups due to the absence of detectable viable bacteria in lung tissue homogenates despite the increased tissue inflammation (H&E staining) and presence of acid-fast bacilli (ZN staining). Scatter plots represent the lung CFU levels and % of lung tissue inflammation within the group of excluded mice (red dotes) (A-C) at 45 days p.i. Abbreviations: CON; control, T2D; type 2 diabetes, CFU; colony forming units, UNT; untreated, MET; metformin, INH; Isoniazid, p.i.; post infection, H&E; Hematoxylin and eosin, ZN; Ziehl-Neelsen.



Figure S5-3: Systemic cytokine/ chemokine profiles. (A) Serum cytokine/ chemokine levels were measured 45 days p.i. and (B) compared against UNT mice. Statistical analysis: \*p < 0.05 by One-way ANOVA followed by Dunnett's multiple comparison test. Results are presented as (A) a heat map and (B) a horizontal bar graph from 7-10 mice per group from two independent pooled experiments. Abbreviations: CON; control, T2D; type 2 diabetes, CFU; colony forming units, UNT; untreated, MET; metformin, INH; Isoniazid, p.i.; post infection.

# Chapter 6

## **General Discussion**

Despite the best international efforts, the battle against TB has become increasingly challenging and falling considerably short of "The End TB Strategy" milestones set by the WHO<sup>8</sup>. The situation is mainly fuelled by the emergence of drug-resistant *Mtb* strains, weak health systems, life style changes and comorbid health conditions such as HIV/AIDS and diabetes; two of the major risk factors for active TB disease. The only current licenced TB vaccine, BCG was developed nearly a century ago. BCG efficacy is highly variable (0-70%) and although it appears to reduce the risk of severe forms of disseminated TB in infants, it fails to stop transmission of pulmonary TB in adults. Furthermore, the failure of BCG to prevent reactivation of LTBI, which is widespread among Mtb/HIV co-infected individuals, underscores the need for a rationally designed anti-TB vaccine that is safe in immunocompromised individuals. Despite the recent advances in immunology and vaccinology, the precise mechanisms underlying the variable protection offered by BCG are unclear and a superior anti-TB vaccine that prevents active TB and LTBI is not available. Unfortunately, against the efforts in reengineering BCG or developing subunit and viralvectored vaccines, most of these candidates have only shown marginally improved protection in animal models of TB. Furthermore, the majority of candidates with the exception of very few (i.e.,  $M72/AS01_{E}^{664}$  and  $H4:IC31^{665}$ ) that have advanced to human clinical trials have not demonstrated superior protection over BCG - a great disappointment to the TB vaccine research community.

#### 6.1 BCG vaccination and reactivation of LTBI in the context of HIV co-infection

Few studies have investigated the impact of conventional BCG vaccination on LTBI reactivation. **Chapter 3** of this thesis explored whether vaccination with standard BCG or improved novel rBCG strains were able to prevent the reactivation of LTBI. While some reports suggest that BCG reduces reactivation of LTBI in both HIV +ve and -ve individuals<sup>603,666</sup>, other studies found no difference between vaccinated and unvaccinated populations in the context of LTBI reactivation<sup>605</sup>. Progressive reduction of CD4<sup>+</sup> T cells is generally believed to be a major factor in LTBI reactivation in HIV co-infected individuals. However, studies identifying precise immune correlates of reactivation are currently lacking. Low-dose (~25 CFUs) *Mtb* infection in NHPs most accurately models the key clinical, histological and microbiological features of human LTBI<sup>667</sup>. Furthermore, simian immunodeficiency virus (SIV) has been used to model HIV infection and induce reactivation of LTBI in NHPs. These studies have also suggested a novel lymphocentric model of TB, in

which *Mtb*-containment during LTBI primarily occurs in lung-draining lymph nodes, with the lung acting primarily as a portal of entry and exit for  $Mtb^{428}$ . In other words, the lymphatics may act as a bridge between the primary entry site in the lung and the site of transmissible pathology following containment failure in draining-lymph nodes. The significant financial and logistical hurdles of NHP research however, preclude their widespread use in dissecting the immunological mechanisms relating to LTBI reactivation

In Chapter 3, a mouse model of latent lymphatic TB<sup>425</sup> was utilised where depletion of CD4<sup>+</sup> T cells using anti-CD4 mAbs leads to a systemic spread of Mtb. The subsequent lung immunopathology and increased organ bacterial loads seen in this model mimic the reactivation dynamics of LTBI seen in HIV<sup>+</sup> individuals. Our findings provide evidence that vaccination with standard BCG or recombinant BCGABCG1419c prevents the reactivation of lymphatic TB in mice independently of vaccine-induced CD4<sup>+</sup> T cells<sup>624</sup>. Although CD4<sup>+</sup> T cells play a critical role during early *Mtb* infection, in a recent NHP study, one-third of the animals co-infected with *Mtb* and SIV were able to prevent reactivation and maintain latency despite the complete absence of lung CD4<sup>+</sup> T cells<sup>429</sup>. The protection from reactivation in these non-reactivators correlates with increased CD8<sup>+</sup> memory T cell (T<sub>CM</sub> and T<sub>EM</sub>) proliferation, granzyme B production, increased levels of iBALT and expanded B cell follicles<sup>429</sup>. More recently, Buscan and colleagues<sup>668</sup>, infected NHPs with a non-T cell depleting SIV mutant (SIV $\Delta$ GY) and showed that mere depletion of CD4<sup>+</sup> T cells was insufficient for the reactivation from LTBI. These studies provide evidence for important roles of non-CD4 immune cells including CD8<sup>+</sup> T and B cells, in containment of LTBI. Despite the statistically significant increase in CD8<sup>+</sup> CD44<sup>+</sup> memory T cell population among i.t. vaccinated mice, we were unable to conclusively demonstrate an increased lung total CD8<sup>+</sup> T cells compared to unvaccinated CD4-depleted mice suggesting that vaccine-induced protection is unlikely to be mediated by CD8<sup>+</sup> T cells in our model. This could be due to the different approaches in reproducing HIV/TB co-infection; CD4<sup>+</sup> T cell depletion vs pathogenic SIV infection in our mouse model vs previous NHP studies, respectively. CD8<sup>+</sup> T cells are involved in the initial control of both SIV and HIV infections. However, only a small portion of CD8<sup>+</sup> T cells that are being expanded following infection are specific for HIV<sup>669</sup>. Interestingly, substantially increased SIV-infected cells were observed within the granulomas of LTBI-reactivators, suggesting that both SIV and *Mtb* synergise and heighten the SIV and TB disease pathology which inversely corelates with CD8<sup>+</sup> T cell numbers. It has been speculated that numerically and functionally augmented CD8<sup>+</sup> T cell responses repress SIV replication within tuberculous granulomas among nonreactivators in SIV-co-infected NHPs<sup>429</sup>. However, this does not explain how BCG-vaccination prevents the reactivation of lymphatic LTBI in mice.

Although we did not further investigate which immune mechanisms prevent the reactivation from LTBI in our model, existing evidence suggest that B cells and innate immune cells also play a crucial role in developing an optimum anti-*Mtb* immune response. Naïve and memory B cells have been found in the granulomas and lesions of Mtb-infected human lungs where they act as APCs presenting Mtb antigens to bystander T cells and secreting cytokines and Mtbspecific antibodies<sup>670</sup>. Similarly, NHP studies have confirmed the presence of B cells that actively produce Mtb-specific IgG antibodies not only within lung granulomas but also in thoracic lymph nodes<sup>671</sup>. Furthermore, B cell depletion using the anti-human CD20 chimeric monoclonal antibody, rituximab, has led to significantly higher bacterial counts in lung granulomas in NHPs and altered cytokine profiles indicating that B cells can modulate the anti-TB immune response, at least in the acute phase of the infection<sup>672</sup>. In the NHP LTBI model, the non-reactivators had formed increased and well-organised iBALT containing CD20<sup>+</sup> B cells proximal to granulomas<sup>429</sup>, strengthening the hypothesis that B cells may have a protective role in LTBI. In addition, B cell deficiency often leads to a neutrophil-dominated lung infiltration and enhanced IL-17/Th17 responses following Mtb infection<sup>673</sup>. Furthermore, BCG vaccine-induced Th1 responses in B cell-deficient mice were significantly diminished due to an impaired DC migration to draining-lymph nodes, suggesting a pivotal role of B cells in vaccine-induced T cell responses<sup>673</sup>.

Vaccines such as Tetanus-diphtheria-acellular pertussis (Tdap) and tetanus-diphtheria (Td) provide long-lasting humoral immunity against diphtheria and tetanus<sup>674</sup>. This long-term protection is believed to be mediated by memory B cells that readily recognise antigen upon infection and their rapid differentiation into antibody producing plasma cells, thereby increasing systemic antibody levels<sup>675</sup>. Sebina *et al.* have previously reported that BCG-vaccinated healthy individuals in low endemic TB areas had significantly higher peripheral BCG-specific memory B cells compared to unvaccinated healthy people and these responses can last for up to 45 years<sup>676</sup>. In contrast, neonatal BCG vaccination had a limited overall impact on T and B lymphocytes among Danish infants<sup>677</sup>. However, multiple human, mouse and NHP studies have provided compelling evidence that mucosal, intradermal and subcutaneous vaccination with BCG induces *Mycobacterium* antigen specific antibody titres<sup>678</sup>. The type and function of *Mtb*-specific Abs during active TB and LTBI remains unclear

although emerging evidence suggests divergent Ab effector functions including FcR binding as occurs in opsonisation and antibody-dependent cellular cytotoxicity (ADCC). Hence, understanding the nature of the humoral response to BCG and how this may be exploited to induce optimal protective immunity remains an important research question.

Interestingly, BCG and some other vaccines such as MV have been shown to induce nonspecific immunity thereby reducing overall TB-unrelated mortality in children as reviewed previously<sup>679,680</sup>. As of June 1<sup>st</sup>, 2020, the new corona virus disease (COVID-19) is currently affecting 213 countries and territories with over 6.3 million laboratory confirmed cases and more than 375,000 deaths<sup>681</sup>. With no vaccine or clinically proven effective antiviral drug for the disease, the entire world is struggling to control the COVID-19 pandemic. Splendidly, reduced COVID-19 related morbidity and mortality are being reported in countries that mandate BCG vaccination<sup>682,683</sup>. These non-specific effects of BCG are believed to be achieved by educating the innate immune system, in a concept now known as 'trained immunity'<sup>641</sup>. Whereas classical adaptive memory immune responses are based on clonal expansion of antigen-specific T cells, trained immunity on the other hand relies on reprogramming of innate myeloid cells via stable epigenetic modifications. It is known that BCG vaccination induces NOD-2-dependent epigenetic and metabolic reprograming including H3K4 trimethylation in monocytes, leading to increased production of pro-inflammatory cytokines<sup>684</sup>. BCGvaccinated severe combined immunodeficiency (SCID) mice also showed significantly increased survival following a lethal dose of Candida albicans infection, further confirming that the vaccine-induced protection was independent of adaptive immune response<sup>684</sup>. Significantly altered DNA methylation patterns in immune-related pathways have also been observed in cells isolated from responders (BCG-vaccinated individuals with enhanced Mtb containment within macrophages). These results suggest a relationship between BCG-induced epigenetic reprogramming of innate immune cells and enhanced efficacy of anti-*Mtb* immunity in BCG-vaccinated individuals<sup>685</sup>. An elegant study by Kaufman and colleagues has recently provided mechanistic evidence that BCG-educated haematopoietic stem cells (HSCs) generate epigenetically modified macrophages which provide significantly better protection against *Mtb* in vivo<sup>686</sup>. Moreover, these findings indicate that BCG vaccination may have imprinted a unique 'memory-like' phenotype into these APCs, altering the rapidity and magnitude of the immune response.

Collectively these data suggest that BCG- and BCG $\Delta$ BCG1419c-induced B cell and 'trained' innate immune responses may be responsible for mediating the *Mtb* containment that we observed within the draining lymph nodes of vaccinated mice (Chapter 3). In our studies, the intratracheal and subcutaneous immunisation routes offered functionally similar but anatomically distinct protective immunity. Further studies could explore these differences and identify the role lymph node microenvironment in controlling these protective immune responses. Using modern imaging and next generation molecular techniques, such as Imaging Mass Cytometry and high throughput RNAseq, respectively, the complex cellular morphology and tissue architecture of lymph nodes and the gene expression and epigenetic modification patterns of each cell subset could be analysed. This will allow a thorough investigation of LTBI reactivation in HIV<sup>+</sup> and other immunocompromised individuals such as diabetics.

# 6.2 New and improved anti-TB vaccines and adjunctive HDTs in the context of comorbid diabetes

As a consequence of intrinsic immune defects caused mainly by a hyperglycaemic environment, people with diabetes are generally more susceptible to infectious agents and to develop severe disease outcomes including in TB. Interestingly, BCG vaccination has been shown to be a promising treatment for a number of diseases including organ-specific autoimmune type 1 diabetes (T1D). A long term follow up study revealed that BCG-vaccinated T1D patients had significantly improved glycaemic control (as reflected by reduced HbA1c levels compared to the placebo group)<sup>644</sup>. In NOD mice, multiple BCG boosts were required to prevent or improve T1D <sup>687</sup> whilst BCG is commonly given as a single dose for humans at birth. In addition, neonatal BCG vaccination prior to the onset of diabetes had no beneficial effects implying that BCG may only be effective when administered during the development of the disease<sup>688</sup>.

Studies in NOD mice given multiple boost of BCG were able to prevent and improve diabetes via rescue and/or regeneration of pancreatic beta cells. In contrast, it has been proposed that in human T1D, BCG induces aerobic glycolysis which increases the uptake and utilization of glucose while reducing the oxidative phosphorylation. The resulting lower blood sugar levels were therefore independent of insulin production<sup>644</sup>, suggesting a possible applicability to other forms of hyperglycaemia such as seen in T2D. Furthermore, increased glycolysis was observed

in BCG-trained innate monocytes as a direct consequence of chromatin remodelling via histone modifications<sup>660</sup>.

The impact of BCG immunisation on glucose metabolism in human T2D or animal models has not be investigated. While it would have been ideal to investigate the impact of BCG vaccines on metabolic pathways, we did not measure the blood glucose or other metabolite levels after BCG vaccination or *Mtb* infection **(Chapter 4 & 5)**. However, the rapid weight loss and increased bacterial persistence we observed in T2D mice following BCG vaccination contradicts the beneficial effects seen in BCG-vaccinated NOD mice. In addition, BCG vaccination also led to an epigenetic de-methylation and increased mRNA expression of a number of T<sub>reg</sub> signature genes<sup>644</sup>; an indication of T cell tolerance which could be beneficial during the chronic phase of *Mtb* infection. In contrast, in our study BCG-vaccinated T2D mice had the highest number of reactive lung T cells, including IFN $\gamma$  and IL-17 producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells and elevated lung inflammation among all vaccinated diabetic mice. We speculate that this was due to an ongoing immune response as a result of higher persistence of BCG. The increased lung bacterial persistence at 60 days following vaccination can be attributed to the mucosal delivery of vaccines in our study compared to more traditional parenteral BCG administration described in other studies.

While diminished antigen recognition, a delay in adaptive immune priming and impaired killing mechanisms by innate immune cells are characteristics of diabetes-associated immune dysfunction (**Chapter 4** and <sup>525,689</sup>), recent findings on bioenergetic pathways of host-immune cells in TB are suggestive of potential avenues as to how immunometabolism may play a pivotal role in increased *Mtb* infection<sup>690</sup>. It has been shown that *Mtb* induces a 'Warburg-like effect' in infected lungs involving the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) pathway primarily characterised by a metabolic shift towards increased glycolysis<sup>690</sup>. This shift promotes a rapid pro-inflammatory response against microbes which can be beneficial for early control of infectious diseases in immune-competent individuals. Whereas, increased reliance on aerobic glycolysis in T2D can cause excessive pro-inflammatory cytokine production which may have associated with augmented lung immunopathology seen in diabetic mice<sup>691,692</sup>. It is therefore important to recognise that a fine balance between pro- and anti-inflammatory immune responses is indispensable for the control of *Mtb* infection. In addition to the induction of glycolysis, synthesis of large lipid bodies from glycolytic intermediates which accumulate in macrophages provide a nutritional niche for the survival of *Mtb*<sup>692</sup>. Distinct lipid profiles

observed in comorbid TB/DM patients provide a correlation between increased TB susceptibility and plasma lipid levels<sup>693</sup>.

Moreover, in Chapter 4, using two experimental rBCG strains containing immunodominant *Mtb* antigens (BCG::RD1 and BCG::RD1 ESAT-6 Δ92-95), we have been able to achieve near sterile immunity against aerosol Mtb infection in both non-diabetic and diabetic mice. As expected, standard BCG vaccination did not substantially improve the TB disease outcome. While ESX-1-expressing strains had induced both adaptive and innate immune responses in non-diabetics via cytosolic immune signalling<sup>590</sup>, the enhanced anti-TB protection in rBCG vaccinated diabetic mice was unlikely to be mediated by vaccine-induced T cells. Our data suggests that vaccination with BCG strains failed to rescue the defective co-stimulatory marker expression of diabetic APCs and did not enhance T cell proliferation in vitro. Instead, rBCG vaccinated lungs displayed both numerically and functionally augmented-innate antigen presenting macrophages and dendritic cells. Higher expression of intracellular iNOS in lung APCs and increased NO production upon in vitro Mtb infection suggest that the superior anti-TB protection was largely mediated by rBCG-induced innate immune responses. Interestingly, using an in vitro experimental model, Bekkering and colleagues have shown that BCG-trained human primary monocytes produced increased cytokines and bactericidal ROS upon the restimulation with LPS<sup>694</sup>. However, it remains unclear how the ESX-1-expressing strains induce a superior innate immune response against TB compared to standard BCG.

Although the induction of innate immune responses is generally considered to be independent of priming stimuli, interestingly, different inducers of trained immunity have led to distinct functional and phenotypic changes in various innate cells<sup>694,695</sup>. Therefore, it is possible that rBCG strains that contain *Mtb*-derived genes may induce distinct epigenetic alternations in innate immune cells, including APCs and NK cells, resulting in a more robust immune response against *Mtb*. Pathogenic *Mtb* and *Mtb* products have been shown to act on host epigenetic factors via DNA methylation, histone modifications and miRNA regulation<sup>696</sup>. It would therefore be necessary to study the effect of rBCG strains on innate cells to understand how epigenetic-induced memory response could be beneficial against pulmonary *Mtb* infection not only in immunocompetent individuals but also in people with immunosuppressive health conditions, such as T2D. However, it would also be warranted to investigate whether this improved innate anti-TB protection was independent of ESAT-6-mediated cytosolic

translocation as both BCG::RD1 and BCG::RD1 ESAT-6  $\Delta$ 92-95 had similar responses, despite the latter strain being unable to access the cytosol<sup>56</sup>.

In addition to the development and improvement of novel and existing anti-TB vaccines, respectively, the use of HDTs is now considered to be crucial to meet the WHO End TB Strategy milestones<sup>697</sup>. While conventional anti-TB drug regimens are effective, poor treatment adherence and long-term conventional mono-therapy often lead to drug tolerance and resistance, thereby significantly contributing to the global TB disease burden. In Chapter 5, we investigated the therapeutic potential of MET; an AMPK-activating anti-diabetic drug against Mtb infection in both non-diabetic and diabetic mice. In line with some of the retrospective human studies where MET prescription was associated with lower incidence of active TB among comorbid patients<sup>657,698</sup>, oral MET therapy (500 mg/kg) significantly reduced the lung Mtb burden in T2D mice. Whether this protection against TB in T2D mice was a consequence of MET-regulated systemic glucose levels remains to be determined<sup>699</sup>. In a recent study, Singhal and colleagues found that MET inhibits the growth of both drugsusceptible and drug-resistant Mtb strains by selectively inducing mitochondrial ROS production in an AMPK-dependent manner<sup>655</sup>. Interestingly, higher systemic levels of advanced glycation end products (AGEs) have been detected in TB/DM comorbid patients, and significantly diminished levels of receptor for AGE (RAGE) ligands were associated with MET therapy<sup>700</sup>. In addition, MET was also found to inhibit AGE-induced pro-inflammatory responses (i.e., IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) and promote anti-inflammatory cytokines including IL-10 from CD206<sup>+</sup> M2 macrophages through suppression of the RAGE and NF<sub>kB</sub> pathway<sup>701</sup>. Furthermore, MET was also shown to induce autophagy in comorbid patients<sup>702</sup>. Collectively, these findings support the beneficial role of MET associated with reduced lung inflammation and improved disease outcome in T2D mice.

More strikingly, data we obtained from non-diabetic mice that received the same therapeutic dose of MET showed a significantly increased lung *Mtb* burden suggesting impaired anti-TB immunity. *Mtb* activates mTOR, and direct or indirect inhibition of mTOR has resulted in diminished Th1 cytokine production in both *in vivo* and *in vitro* models of *Mtb* infection<sup>661</sup>. Recently, it was also shown that MET inhibits type 1 interferon responses in healthy human CD4<sup>+</sup> T cells independently of AMPK activation or mTOR inhibition following anti-CD3e/CD28 stimulation<sup>703</sup>. In our study, we did not observe any significantly elevated systemic anti-inflammatory cytokine levels nor was the cellular immune response assessed. More recently it
has been shown that ex vivo BCG-trained monocytes from healthy individuals who were receiving increasing doses of MET in the previous 6 days had a temporarily decreased induction of pro-inflammatory IL-6 and TNF levels upon stimulation<sup>660</sup>. Furthermore, MET also abrogated the  $\beta$ -glucan induced trained immunity against *C. albicans*<sup>704</sup>. These findings imply that MET could alter the epigenetic modifications associated with trained immunity by inhibiting the mTOR-HIF-1a pathway. It is currently not known how and if MET-mediated mTOR inhibition impacts on unvaccinated or untrained immune systems. Lastly, as briefly discussed in Chapter 4, a perturbed gut microbiota influences mucosal immune responses in the lungs via the 'gut-lung axis'. Antibiotic-mediated dysbiosis in intestinal microbiota has been linked to early susceptibility to *Mtb* infection and bacterial dissemination in mice<sup>365,366</sup>. An altered gut microbiota has been reported in healthy individuals as early as 24 hours after commencement of MET treatment<sup>662</sup> and also in healthy C57BL/6 mice treated with MET for 30 days<sup>663</sup>. Intriguingly, comparative analysis of altered microbiome profiles of MET treated healthy mice demonstrated a strong and a weak positive correlation with pre-diabetes and T2D, respectively, suggesting that MET may at least induce a gut environment favouring prediabetes<sup>663</sup>. Overall, the therapeutic potential of MET against TB is promising and exciting. The 'Metformin for TB/HIV Host-directed Therapy' (METHODS) trial will be the first prospective clinical trial evaluating the efficacy of MET on lung function, severity of lung involvement and HIV viral load in addition to the proportion of patients with a negative sputum culture at 2 months post-treatment (R34-AI124826-01)<sup>705</sup>. However, our findings on nondiabetic mice suggest that further pre-clinical studies are warranted to investigate the true impact of MET on the immune system and the microbiota of healthy people prior to assessing its suitability to use as an HDT or an adjunctive TB therapy in humans.

In conclusion, this thesis provided experimental evidence: (i) that BCG vaccination could prevent latent-like TB infection in a murine model independent of classical CD4<sup>+</sup> T cells which was previously thought to be indispensable for anti-TB immunity; (ii) that experimental recombinant BCG vaccines containing *Mtb* antigen systems (ESX-1; RD1) confer near sterile immunity in both non-diabetic and immunosuppressed diabetic mice despite these strains having previously been deemed unsuitable for human use; and (iii) that anti-diabetic MET has divergent effect on TB disease in diabetic versus non-diabetic mice. The findings from **Chapter 3** and **4** emphasise the importance of the innate immune response against TB. This thesis together with emerging data from the TB field, suggest that understanding TB-specific epigenetic landscapes and harnessing the effects of vaccine-induced trained immunity could

inform the development of next generation anti-TB vaccines and better therapeutic regimens. In this study, the significance of reconsidering alternate routes of BCG administration has also been demonstrated; particularly intratracheal or other mucosal routes. Furthermore, this thesis reveals that incorporating *Mtb*-derived antigens into new vaccine candidates elicits a greater mucosal immune response leading to a better protection against TB. The MET study (**Chapter 5**) revealed that adjunct HDT is unlikely to be a 'one-size-fits-all' approach and will require further studies investigating the shortcomings of HDTs including potential adverse effects in diseased versus healthy individuals. Further research will be needed to fill the knowledge gaps described and accelerate the development and implementation of new and improved TB vaccines as well as drug regimens making a pronounced contribution to the global TB elimination efforts.

## Chapter 7

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# **Chapter 8**

# Appendices

This chapter 'Appendices' contains previously unlisted PhD-related publications that have been co-authored during the candidature.

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## 8.1 Appendix 1: A systemic approach to simultaneously evaluate safety, immunogenicity, and efficacy of novel tuberculosis vaccination strategies

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## IMMUNOLOGY

## A systematic approach to simultaneously evaluate safety, immunogenicity, and efficacy of novel tuberculosis vaccination strategies

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Tuberculosis (TB) is the deadliest infectious disease worldwide. Bacille-Calmette-Guérin (BCG), the only licensed TB vaccine, affords variable protection against TB but remains the gold standard. BCG improvement is focused around three strategies: recombinant BCG strains, heterologous routes of administration, and booster vaccination. It is currently unknown whether combining these strategies is beneficial. The preclinical evaluation for new TB vaccines is heavily skewed toward immunogenicity and efficacy; however, safety and efficacy are the dominant considerations in human use. To facilitate stage gating of TB vaccines, we developed a simple empirical model to systematically rank vaccination strategies by integrating multiple measurements of safety, immunogenicity, and efficacy. We assessed 24 vaccination regimens, composed of three BCG strains and eight combinations of delivery. The model presented here highlights that mucosal booster vaccination may cause adverse outcomes and provides a much needed strategy to evaluate and rank data obtained from TB vaccine studies using different routes, strains, or animal models.

#### INTRODUCTION

Tuberculosis (TB) kills more people annually than any other infectious disease (1). Currently, live-attenuated Bacille-Calmette-Guérin (BCG) is the only licensed TB vaccine for human use. Although it has good ability to prevent extrapulmonary forms of TB in young children such as TB meningitis and miliary TB, the capacity of BCG to protect against pulmonary TB, the most common and transmissible form of the disease, is variable (2). Nonetheless, BCG continues to be widely administered intradermally in many TB endemic countries. To reach the World Health Organization's 2035 The End TB Strategy goal of reducing the incidence of TB by 90% (1), and to stop the rise of multidrug-resistant strains of Mycobacterium tuberculosis (Mtb), the causative agent of TB, more efficacious vaccines are needed. Over the past two decades, several new TB vaccine candidates have been developed and are currently undergoing clinical testing. In addition to subunit vaccines composed of selected dominant Mtb antigens (3, 4), live recombinant BCG strains and attenuated Mtb strains appear to be among the most promising vaccine candidates (5). It has also recently become apparent that the route of vaccine administration and whether the vaccine requires a booster application critically influence stage-gating decisions during the clinical development of novel TB vaccines. For example, a recently completed trial in South Africa that assessed the prevention of Mtb infection in neonatally BCG-vaccinated adolescents via booster immunization with the subunit vaccine candidate H4:IC31 or BCG, demonstrated a significant reduction in sustained QuantiFERON conversion by BCG revaccination (4). Although these results contrast with an earlier

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

BCG revaccination trial in Brazil (6), they warrant further investigations into the importance of BCG booster application and whether this should occur by altering the route of administration or by engineering BCG to express additional antigens. While animal models can contribute to systematically answer these questions, there remains a discrepancy between preclinical studies, where immunogenicity and efficacy parameters are often prioritized, and clinical practice, where safety and efficacy are the dominant considerations.

Mtb has evolved various mechanisms to modulate and evade host immune functions such as phagolysosome fusion and antigen presentation to survive and replicate within macrophages. The ESX (type VII) secretion systems, while nonessential for bacterial growth, appear to be necessary for conferring virulence (7). Encoded within the region of difference 1 (RD1), early secreted antigenic target (ESAT)-6 secretion system-1 (ESX-1) is thought to play a role in phagosome disruption and activation of cytosolic pathogen recognition receptors. Genomic deletion of RD1 results in attenuation of Mtb, further implicating ESX-1 in virulence (8, 9). All Mycobacterium bovis BCG strains, including those currently licensed for human use, lack RD1. Although an experimental strain of BCG reconstituted with an extended RD1 region from Mtb (BCG::RD1) has been demonstrated to reduce Mtb replication, it maintains proliferative capacity in lung and splenic tissues of immunocompromised mice, rendering this strain too virulent for human vaccine use (10). However, we have recently shown that ESX-1-mediated cytosolic contact of ESAT-6 is required to activate protective immune responses against TB (11). In this study, rapid secretion of interferon- $\gamma$  (IFN- $\gamma$ ) only occurred when full-length ESAT-6 was secreted via ESX-1 but not when a C-terminal truncated version of ESAT-6, unable to reach the host cell cytosol, was expressed (BCG::RD1 ESAT-6 Δ92-95). Furthermore, improved protection against TB conferred by intravenous vaccination with BCG::RD1 at day 45 was lost when mice were vaccinated with BCG::RD1 ESAT-6 ∆92–95 (11). These findings support increasing evidence that initiation of cytosolic contact, as conferred by many recombinant BCG strains, improves protection (12).

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In murine models, protection against TB generated through parenteral BCG administration relies on the induction of T helper 1 (T<sub>H</sub>1) CD4<sup>+</sup> memory lymphocytes (13, 14). However, many of the vaccine-induced lymphocytes lack the necessary mucosal-homing chemokine receptors to migrate into pulmonary tissue. This results in a delay in activation and recruitment of TB-specific effector (T<sub>EM</sub>) and central (T<sub>CM</sub>) memory lymphocytes to the lungs, allowing *Mtb* to infiltrate macrophages, proliferate, and establish chronic infection. In addition, a third type of memory T lymphocytes, largely CD69<sup>+</sup>CD103<sup>+</sup> cells, called tissue-resident memory T lymphocytes (T<sub>RM</sub>), have recently been found to take permanent residence in nonlymphoid tissues. These cells are ideally situated in portals of entry, allowing them to mount timely recall responses unlike their circulating counterparts (*15, 16*) and hence present as a possible cellular target for vaccination strategies.

Several studies have demonstrated that mucosal administration of BCG results in the generation of significant numbers of protective anti-TB airway  $T_{RM}$  (17–19). This suggests that the current protocol of administering vaccines parenterally needs to be reconsidered, with mucosal vaccination strategies appearing more efficacious (20). This also agrees with findings by Calmette and Guérin (21), published in 1931, that BCG vaccination administered via the oral route demonstrates good results. It has also been shown that T<sub>RM</sub> can be generated in a number of peripheral barrier tissues (skin and mucosa) using the "prime and pull" booster vaccination method (22). In this strategy, memory lymphocytes are systemically primed using a traditional subcutaneous vaccination, followed by topical administration of inflammatory agents such as chemokines to the desired peripheral tissue. However, this strategy appears to be dependent on local antigen presence for generation of T<sub>RM</sub> within the lung. Thus, a live vaccine capable of initially replicating in the host and maintaining antigenic stimulation may be more effective.

Despite these many efforts to develop more efficacious TB vaccines, intradermal BCG vaccination remains the gold standard. To facilitate preclinical decision-making and stage gating, here we used a systematic and comprehensive approach to investigate if a combination of recombinant BCG (rBCG) strains that express Mtb-specific antigens (BCG::RD1 and BCG::RD1 ESAT-6 A92-95), heterologous route vaccination [intratracheal (IT) and/or subcutaneous (SC)], and booster vaccination strategies can enhance protection against aerosol Mtb challenge and induce T<sub>RM</sub>. While C57BL/6 mice are the most commonly used preclinical small-animal model to study immunogenicity and efficacy in preclinical BCG development (representative of largely resistant healthy humans), SCID (severe combined immunodeficient) mice are traditionally used to assess the safety of live vaccines. To allow comprehensive safety evaluation within the C57BL/6 model itself, we have analyzed clinical, histological, and chemical pathology, such that safety can be evaluated simultaneously with immunogenicity studies. All parameters were compared to the current BCG protocol (represented by BCG::pYUB, a BCG strain containing a gene empty vector). An "empty vector" BCG control has been used to account for any effects that the vector may have on microbial fitness in vivo. In doing so, we developed an empirical model to integrate multiple measurements of safety, immunogenicity, and efficacy to rank each vaccination strategy. Using this approach, we find that (i) the expression of immunodominant antigens from the Mtb genome is the most effective way to enhance efficacy of BCG, (ii) that the mucosal administration of BCG booster doses is correlated with adverse safety outcomes, and (iii) that immunogenicity

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

readouts do not predict efficacy. The model presented here provides a much needed strategy for the TB vaccine community to evaluate and rank data obtained from vaccine studies using different routes, strains, or animal models.

#### RESULTS

The experimental outline and experimental groups are shown in Fig. 1.

#### Vaccine safety

Throughout the vaccine period, animal weight and signs of disease were monitored as clinical indicators of health. Sixty days after prime vaccination, vaccine clearance, lung histology, and serum cytokine profiles were analyzed. These parameters were selected as indicators of either lung or systemic health.

## In vivo persistence of BCG is dependent on the route of vaccination and strain characteristics

The persistence of viable mycobacteria in the lung and splenic tissue of animals following vaccination was investigated as an indicator of local and systemic clearance of the vaccines. Sixty days following prime vaccination, viable bacilli were readily recovered from lung tissue of animals that were IT vaccinated, while minimal colony-forming units (CFU) were recovered from lung tissue of animals that received only SC vaccination(s) (Fig. 2A). Furthermore, IT vaccination resulted in higher CFU numbers in the splenic tissue when compared with SC vaccinated animals (Fig. 2B), indicating that IT delivery may allow for widespread dissemination of bacilli. Only BCG::pYUB and BCG::RD1 persisted in the lung tissue in significant amounts in some groups that received an IT vaccination. The number of bacilli recovered from the lung tissue of BCG::RD1 ESAT-6 Δ92–95-vaccinated mice was not significant, suggesting that this strain may be the most readily cleared from the lungs. The number of bacilli in lung tissues tends to reduce with time in all IT-vaccinated groups (Fig. 2A; -IT versus IT-). On the contrary, viable BCG::RD1 ESAT-6 A92-95 and BCG::RD1 remained in splenic tissue in significant amounts in more groups than BCG::pYUB.

#### *IT vaccination induces inflammatory changes in the lung* To further assess the safety of each vaccination strategy, we assessed

histological changes in lung tissue 60 days after the initial prime vaccination. In line with previous studies (17), hematoxylin and eosin (H&E) staining of lung sections from immunized animals revealed that IT BCG vaccination induced inflammatory changes in the lung parenchyma (Fig. 2C), including the presence of foamy macrophages, lymphocyte, and granulocyte infiltration. While some areas of tissue infiltration in close proximity to bronchioles resembled highly organized structures evocative of inducible bronchus-associated lymphoid tissue (iBALT) (Fig. 2C, green arrow head), other changes included edema and generalized foci of inflammation (Fig. 2C, yellow arrow head). Areas of consolidation and/or increased leukocyte infiltration were considered altered regardless of the pattern of inflammation (Fig. 2C, green and yellow arrow heads). Inflammation was almost completely absent in lung sections of animals that received only SC vaccination(s) and resembled the lung architecture of unvaccinated (naïve) animals (Fig. 2C, red arrowhead). IT vaccination, however, induced widespread inflammatory changes of heterogeneous patterns. Morphometric quantitation was performed, where the percentage of area affected by these changes was calculated (Fig. 2E). Animals vaccinated with BCG::pYUB and BCG::RD1 exhibited the greatest amounts of inflammation, while changes

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**Fig. 1. Schematic representation of vaccination/infection model.** Six- to 8-week-old female naïve C57BL/6 mice were vaccinated via the SC or IT route or left unvaccinated. Twenty days later, animals received a booster vaccination via the same or alternate route or did not receive a booster. During the vaccination period, mice were weighed weekly and assigned daily a clinical health score. Sixty days after prime vaccination, some mice from each group were euthanized, and clearance of bacilli from the lungs and spleen, cellular immune responses in the lung interstitium and airways, lung histology, and serum cytokine/chemokine profiles were studied (vaccine safety and immunogenicity). At day 90 after prime vaccination, remaining mice were aerosol infected with *Mtb* H37Rv. At day 118, *Mtb* colony-forming units (CFU) from lung tissue was enumerated, and lung histopathology was analyzed (vaccine efficacy). Three recombinant strains of BCG were investigated: BCG:::pYUB (standard BCG reconstituted with an empty control plasmid conferring resistance to hygromycin, serving as the control strain), BCG:::RD1 eSAT-6 Δ92–95. Together with unvaccinated mice, this resulted in a total of 25 experimental groups.

observed in animals vaccinated with BCG::RD1 ESAT-6  $\Delta$ 92–95 did not reach significance (Fig. 2, D and E). Unexpectedly, all BCG::pYUBimmunized groups that received at least one IT vaccination demonstrated significant amounts of inflammation. BCG::pYUB also induced the greatest amount of inflammation across all vaccine strategies that involved IT vaccination. Comparison of groups that received a single IT vaccination at different time points suggested that these inflammatory changes were transient, as the percentage of lung involvement tended to reduce with time in BCG::pYUB- and BCG::RD1-vaccinated groups. Although this was not true for BCG::RD1 ESAT-6  $\Delta$ 92–95–vaccinated mice, the changes induced in these animals were not significant at either time point, and thus, the slight increase in inflammatory change observed following BCG::RD1 ESAT-6 Δ92-95 vaccination may be negligible. It is, however, important to note that due to the large number of comparisons and multiple hypothesis testing in this study, individual comparisons among the entire group did not always pass our significance threshold, despite individual comparisons to unvaccinated mice across fewer groups showing statistical significance. Further investigation is needed regarding the different types of inflammation observed and whether pathological changes affect respiratory function or are protective structures that mediate immunity.

## IT administration of booster vaccinations causes a transient state of illness

A single SC or IT vaccination did not cause any noticeable signs of disease, regardless of the vaccine strain administered. Body weights of these animals mirrored those of unvaccinated controls. However, animals that received a prime-boost vaccination strategy, where the booster was administered via the IT route (SCIT and ITTT; see table in Fig. 1), exhibited signs of illness following the booster dose. These animals suffered loss of weight and displayed clinical signs of illness

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

including dyspnea, lethargy, and anorexia, 24 to 48 hours following the IT booster, with some animals euthanized for ethical reasons (three mice in the BCG::pYUB SCIT group and two mice in the BCG::RD1 SCIT group). The SCIT vaccination strategy had the greatest effect on health (Fig. 2F). Although this phenomenon was consistent among all vaccine strains investigated, animals vaccinated with BCG::pYUB developed the most severe clinical signs and suffered the greatest weight loss, while BCG::RD1-vaccinated animals were the slowest to regain weight. Despite this transient state of illness, animals appeared to recover as indicated by weight gain and returned to normal health scores by day 60. Collectively, these results imply that an IT booster vaccination with a live wholeorganism vaccine administered 20 days after prime vaccination may be unsafe.

## Prime-boost vaccinations with BCG::RD1 induce a prolonged systemic inflammatory profile

To determine whether any of the vaccination strategies caused and maintained elevated levels of systemic cytokines, serum samples from vaccinated animals were analyzed for various chemokines and cytokines 60 days following prime vaccination (Fig. 3A). Across all vaccination strategies, a similar pattern was found. While proinflammatory cytokines are expected to increase immediately following vaccination, inflammation should subdue with time. Interleukin-1β (IL-1β), IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) were used as markers of systemic inflammation due to their involvement in pathological hyperinflammatory states such as cytokine storm. None of the BCG::pYUB and BCG::RD1 ESAT-6  $\Delta$ 92–95–vaccinated groups exhibited an increase in systemic levels of IL-1β, IL-6, and TNF- $\alpha$ relative to unvaccinated controls at day 60. However, animals immunized with BCG::RD1 via SCSC and SCIT strategies had significantly elevated levels of IL-1β and IL-6, respectively (Fig. 3B).



**Fig. 2. Vaccine safety.** BCG CFU recovered from (**A**) lung tissue and (**B**) splenic tissue. (**C**) Representative images of iBALT-like structures (green arrowhead), generalized inflammation (yellow arrowhead), and normal lung tissue (red arrowhead) at ×40 magnification. (**D**) Representative H&E staining of lung sections from each experimental group at ×40 magnification. (**E**) Morphometric quantitation of histological changes presented as a percentage of the total lung area. (**F**) Weekly weight changes and health scores. Weight changes (top row) are presented as mean values from two pooled independent experiments (n = 6 to 13 mice per group). Health score results (bottom row) are presented as mean values from one representative experiment (n = 7 mice per group). (A to C) Presented as means ± SEM from two pooled independent experiments (n = 10 mice per group). The statistical significance of differences between each experimental group relative to unvaccinated controls is shown. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001, and \*\*\*\*P < 0.0001. The *P* values were determined using a one-way ANOVA followed by a Dunnett's multiple comparison test.

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020



Fig. 3. Systemic cytokine/chemokine profile and serum IgA levels before *Mtb* infection. (A) Serum cytokines/chemokines 60 days following prime vaccination presented in a heat map. (B) Serum levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  used as measures of systemic inflammation for vaccine safety studies. (C) Serum levels of key T<sub>H</sub>1 cytokines (IFN- $\gamma$ , IL-2, and IL-12p70). (B and C) Presented as means  $\pm$  SEM from two pooled independent experiments (n = 10 mice per group). (D) Serum levels of IgA used as a measure of humoral immunity for immunogenicity studies (n = 3 mice per group). The statistical significance of differences between each experimental group relative to unvaccinated controls is shown. \*P < 0.05 and \*\*P < 0.01. The P values were determined using a one-way ANOVA followed by a Dunnett's multiple comparison test.

#### Vaccine immunogenicity

To assess the immunogenicity of each vaccination strategy, serum cytokine/chemokine profiles, serum immunoglobulin levels, and airway and lung interstitial immune cell composition were analyzed 60 days after prime vaccination.

## Parenteral vaccination is more likely to induce a $T_{\rm H}{\rm 1}$ cytokine milieu in the serum

 $\rm T_{H1}$  effector responses have been implicated in the effective control of Mtb infection. Hence, key  $\rm T_{H1}$  cytokine (IFN- $\gamma$ , IL-2, and IL-12p70) levels in serum 60 days following prime vaccination were analyzed to determine which strategies established a  $\rm T_{H1}$  profile. The only

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

vaccination strategies that maintained significantly elevated levels of IFN- $\gamma$ , IL-2, and/or IL-12p70 were BCG::RD1 -SC, BCG::RD1 SCSC as well as BCG::pYUB -SC and SCIT, respectively. None of the BCG::RD1 ESAT-6  $\Delta$ 92–95–vaccinated groups maintained any of these cytokines at significant amounts (Fig. 3C).

### IT vaccination increased serum immunoglobulin A levels

Immunoglobulin A (IgA) has been associated with enhanced protection against Mtb (23). To determine which strategy induced the greatest humoral response, total serum IgA concentrations were measured 60 days after prime vaccinations. Although vaccination appeared to generate a rise in antibody levels, none of the vaccine

### SCIENCE ADVANCES | RESEARCH ARTICLE

strategies induced statistically significant increases in IgA levels (Fig. 3D). Nonetheless, strategies that included at least one IT vaccination appeared to induce a trend toward stronger IgA response when compared with SC vaccination alone.

## IT vaccination with BCG::RD1 induces significantly increased number of airway ${\rm T}_{\rm RM}$

Since airway T<sub>RM</sub> appears to be vital in protecting against *Mtb* (18) and interstitial T<sub>RM</sub> is thought to replenish airway T<sub>RM</sub> populations (24), phenotypic analysis of lymphocytes found in both compartments was performed. Sixty days following prime vaccination, bronchoalveolar lavage fluid (BALF) was harvested, and lungs were perfused with phosphate-buffered saline (PBS) through the right ventricle of the heart to eliminate lymphocytes that were not associated with pulmonary vasculature. Cells were isolated for flow cytometry to define and quantify the population of memory T lymphocytes generated in response to vaccination. IT vaccination generated increased numbers of both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes within the airways and interstitium when compared with vaccination strategies that only included SC administration(s). Many of these lymphocytes displayed a memory phenotype (CD44<sup>hi</sup>). Most of the memory T cells were peripheral tissue-homing lymphocytes (CD44<sup>hi</sup>CD62L<sup>lo</sup>), although substantial populations of central memory T lymphocytes (CD44<sup>hi</sup>CD62L<sup>hi</sup>) were also observed (Fig. 4A and fig. S1A). The characteristic coexpression of CD69 and CD103 within the CD44  $^{\rm hi}\rm CD62L^{\rm lo}$ population was used to differentiate between T<sub>EM</sub> and T<sub>RM</sub> (Fig. 4B and fig. S1B). When all 24 groups were statistically compared with unvaccinated mice, only strategies that included the IT administration of BCG::RD1 induced a significant population of airway T<sub>RM</sub> (Fig. 4B). However, both BCG::pYUB and BCG::RD1 stimulated a significant T<sub>RM</sub> response in the lung interstitium (fig. S1, A and B), while BCG::RD1 ESAT-6  $\Delta$ 92–95 failed to do so in either compartment. Combined heterologous route vaccination strategies appeared to reduce the generation of interstitial  $T_{RM}$ , as the  $T_{RM}$  population in the SCIT and ITSC groups did not reach significance.

Analysis of  $T_{\text{RM}}$  based on the expression of CD4 and CD8 revealed that CD8<sup>+</sup> cells generally accounted for the largest proportion of T<sub>RM</sub> established in airways following vaccination (Fig. 4C). A different pattern was observed in the interstitium, where BCG::pYUBimmunized mice developed a larger population of  $\mathrm{CD4}^{\scriptscriptstyle +}\,\mathrm{T}_{\mathrm{RM}}$  , while BCG::RD1-immunized animals developed a higher proportion of CD8<sup>+</sup> T<sub>RM</sub> (fig. S1C). Furthermore, double-negative T cells (CD4<sup>-</sup>CD8<sup>-</sup>) appeared to account for a smaller proportion of the T<sub>RM</sub> population in interstitium compared with airways. Lymphocytes were also stained for the CD4 major histocompatibility complex (MHC) peptide tetramer derived from the immunodominant Mtb antigen ESAT-6 (QQWNFAGIEAAASA). Only groups vaccinated with a single dose of BCG::RD1 via the IT route established a significant population of tetramer<sup>+</sup> cells in both the airways and lungs (Fig. 4D and fig. S1D). ITIT vaccination with BCG::RD1 appeared to markedly reduce tetramer<sup>+</sup> cells in both compartments. In line with previously published data regarding lung  $T_{RM}$  retention (25), comparison of single IT vaccinations with BCG::RD1 at different time points (40 days, -IT; 60 days, IT-) also suggested that the population of tetramer<sup>+</sup> T<sub>RM</sub> induced by IT vaccination may not persist in the long term. From our data, it appears that a single IT vaccination generated the most dynamic and robust T lymphocyte response in the airways and lung interstitium at day 60. Vaccination with BCG::RD1 ESAT-6 Δ92-95 did not induce tetramer<sup>+</sup> cells. It has been demonstrated that secretion of mycobacterial antigens is essential for their efficient presentation

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

to CD4<sup>+</sup> T cells (26, 27). However, the situation in which biologically nonfunctional ESAT-6 molecules are secreted such as ESAT-6  $\Delta$ 92–95 or the previously used ESAT-6  $\Delta$ 84–95 constructs opens new interesting questions (28). Given that MHC-II peptide loading is not meant to involve cytosolic contact and that the C-terminal truncation of ESAT-6 does not overlap with the CD4<sup>+</sup> T cell epitope tested, these results warrant further investigation into the role of ESAT-6-mediated cytosolic translocation for generation of ESAT-6-specific T cells.

#### Vaccine efficacy

To determine the effect of each vaccination strategy on *Mtb* clearance and lung pathology, *Mtb* challenge experiments were conducted 90 days after prime vaccination.

## All vaccination strategies and strains conferred similar levels of protection against Mtb burden 28 days after infection

It has previously been reported that IT vaccination with BCG significantly reduces the bacterial burden at 45 days following *Mtb* challenge (at which point bacterial burden plateaus) when compared to SC vaccination (17). We investigated whether ESAT-6–expressing rBCG and/or heterologous route prime-boost vaccination strategies would induce increased protection early after *Mtb* infection. Ninety days after the priming vaccination, mice were aerogenically challenged with an ultralow to low dose of *Mtb* H37Rv (5 to 80 CFU) and were euthanized 28 days later. All vaccination strategies demonstrated significant reductions in *Mtb* CFU (1 to 1.5 log) when compared with unvaccinated controls (Fig. 5A). However, when compared holistically, there were no significant differences between experimental groups, suggesting that neither the rBCG strains tested nor the heterologous route prime-boost vaccination strategies led to increased protection at an earlier time point.

## Vaccination with rBCG strains reduces lung pathology across vaccination strategies

Twenty-eight days following *Mtb* infection, histopathology analysis of H&E-stained lung sections and morphometric quantitation were performed (as described in the "Vaccine safety" section above). While some organized immune tissue changes reminiscent of tertiary lymphoid structures induced by IT vaccination were maintained, pathological changes such as pneumonia, granulomatous-like inflammation, and diffuse alveolar damage were prominent (Fig. 2E). Whether lung pathology in IT BCG–vaccinated groups are attributable to changes induced by the IT vaccination itself (Fig. 5, B and C) requires further investigation. Across all groups, animals that received BCG::RD1 or BCG::RD1 ESAT-6  $\Delta$ 92–95 tended to suffer the least amount of lung pathology.

### Vaccine empirical integrated model

To simultaneously evaluate experimental parameters of vaccine safety, immunogenicity, efficacy, and overall performance, we developed an integrated approach, the vaccine empirical integrated model (VEIM) (Fig. 6 and VEIM methods; Eqs. 1 to 3). VEIM considers (i) efficacy as the combined effect of reduced *Mtb* CFU and lung pathology after challenge; (ii) immunogenicity as the combined effect of T<sub>RM</sub> in BALF and lung, equally weighted levels of IL-2, IL-12p70, and IFN- $\gamma$ , as well as IgA after vaccination; and (iii) safety as the combined effect of clinical score, weight reduction, lung and spleen bacterial burden, histopathological changes to the lung, and equally weighted levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  after vaccination. For comparison, BCG::pYUB SC- was considered a representation of the current BCG strategy used clinically. Parenteral (SC- and SCSC) administration

## SCIENCE ADVANCES | RESEARCH ARTICLE



**Fig. 4. Airway immune cell profiles following vaccination.** Sixty days following prime vaccination, animals were euthanized, and BALF was harvested for FACS analysis. **(A)** Representative FACS plots and enumeration of CD44<sup>h1</sup> T lymphocyte subsets  $T_{CM}$ ,  $T_{EM}$ , and  $T_{RM}$ . Indicated significance is of total memory T lymphocyte numbers. **(B)** Representative FACS plots and quantification of CD4<sup>+</sup>CD8<sup>-</sup> and CD4<sup>+</sup>CD8<sup>-</sup> (DN)  $T_{RM}$ . Indicated significance is of total  $T_{RM}$  numbers. **(C)** Total numbers of CD4<sup>+</sup>, CD8<sup>+</sup>, and DN  $T_{RM}$ . **(D)** Representative FACS plots showing the gating strategy to enumerate tetramer<sup>+</sup> cells and total numbers of tetramer<sup>+</sup> T\_{RM}. **(E)** Proportion of tetramer<sup>+</sup> and tetramer<sup>-</sup> CD4<sup>+</sup> T\_{RM} are also shown. (A to D) Results are presented as means  $\pm$  SEM from two pooled independent experiments (*n* = 5 to 10 mice per group). The statistical significance of differences between each experimental group relative to unvaccinated controls is shown. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. The *P* values were determined using a one-way ANOVA followed by a Dunnett's multiple comparison test.

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020



**Fig. 5. Vaccine-induced protection and pulmonary pathology following** *Mtb* **infection.** (A) Twenty-eight days following *Mtb* **infection**, animals were sacrificed, and the lungs were assessed for numbers of viable *Mtb*. (B) H&E-stained lung sections were analyzed for histopathology. (C) Representative H&E staining of lung sections from each group at ×40 magnification. (A and B) Presented as means  $\pm$  SEM from two pooled independent experiments. (A) n = 7 to 12 mice per group and (B) n = 6 to 8 mice per group. The statistical significance of differences between each experimental group relative to unvaccinated controls is shown. Scale bar, 600 µm. \*P < 0.05, \*\*P < 0.01, \*\*P < 0.001, and \*\*\*P < 0.001. The *P* values were determined using a one-way ANOVA followed by a Dunnet's multiple comparison test.

of the more virulent BCG::RD1 ESAT-6 Δ92-95 outperformed BCG::pYUB on safety, while BCG::RD1 SC- (previously deemed too unsafe for use in humans) ranked sixth (Fig. 6A). BCG::pYUB SC- appears to be one of the least efficacious vaccine strategies (Fig. 6C), while a single dose of BCG::RD1 obtained the greatest score for protection. Notably, vaccine strategies that ranked in the top half for efficacy were all rBCGs, while all BCG::pYUBvaccinated groups ranked in the bottom half. This supports a model where the expression of key Mtb virulence factors is the most effective way of enhancing vaccine efficacy out of the strategies investigated. Furthermore, some of the most efficacious strategies ranked at the bottom for immunogenicity (Fig. 6B). Using our model, it appears that the most efficacious and safe vaccine strategies are single or double SC doses of BCG::RD1 or BCG::RD1 ESAT-6 Δ92-95 (Fig. 6D and table S2). When efficacy, safety, and immunogenicity were combined into a single rank (fig. S3A), our integrated data analysis indicates that two doses of BCG::RD1 given SC appear to be the most favorable strategy overall (Fig. 6D). To account for the fact that vaccine safety is always paramount and is more important than efficacy and immunogenicity, we also performed three alternate VEIM analyses, in which safety, immunogenicity, and efficacy were weighted differently to calculate the overall rankings (fig. S3B). In addition to equal ranking, the following situations were modeled: (i) safety as twice as important as efficacy and immunogenicity, (ii) both safety and efficacy as twice as important as immunogenicity, and (iii) efficacy in-between safety (as most important) and immunogenicity (as least important). In all scenarios BCG::RD1

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

SCSC remained the most favorable vaccination strategy, followed by single or double IT vaccinations with BCG::RD1. These findings suggest that BCG strains expressing *Mtb* antigens should be reevaluated in the context of mucosal and/or booster application and demonstrate that VEIM can be used in a versatile manner. VEIM also illustrates a disconnection between immunogenicity and protection of BCG strains more broadly (Fig. 6D), highlighting deficiencies in the understanding of true correlates of vaccine-induced protection against TB.

### DISCUSSION

It is widely accepted that studies evaluating novel TB vaccine strategies should include data regarding safety, immunogenicity, and efficacy. However, preclinical evaluation of new TB vaccines is biased toward immunogenicity and/or efficacy, and there remains no standard approach to integrating each of these parameters to determine how novel vaccine strategies perform overall in comparison to standard BCG. To address this, we used a systematic approach to evaluate and compare 24 vaccination regimens in the commonly used preclinical C57BL/6 mouse model. Upon completion of routinely performed data collection, we developed a model, VEIM, to rank vaccine strategies based on overall performance and simultaneous comparison of protection, immunogenicity, and safety. Our results show that expression of *Mtb*-specific immunodominant antigens is the most effective way to enhance efficacy of BCG and that mucosal BCG booster delivery correlates with adverse safety

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Immunogenicity

С

Efficacy

Fig. 6. Vaccine empirical integrated model. Simultaneous comparison of safety, immunogenicity, and efficacy through integration of measurements into single measures of their summed effects (Eqs. 1 to 3 and Materials and Methods). (A to C) Vaccine strategies ordered from right to left based on increased safety, immunogenicity, or efficacy. (D) Vaccines plotted on overall performance of immunogenicity versus efficacy. The size of circles (D) is scaled to safety effect, where the safest strategy is represented by the largest circle. The overall ranking of each vaccine strategy is indicated by the number next to the circle and as a bar graph.

outcomes. VEIM also provides a tool to evaluate and rank data obtained from different TB vaccine studies.

SCIENCE ADVANCES | RESEARCH ARTICLE

Safety

в

Α

5

0

SC administration of BCG provides some, but variable, protection against Mtb infection in murine models (14). Although T<sub>H</sub>1 immune responses have been implicated for protection, the exact immune response(s) that are responsible for reducing bacterial replication remain unknown (29). Following Mtb infection,  $T_{RM}$ , a subset of memory T lymphocytes that take residence in peripheral tissue compartments has been observed in the lung tissue (30). It has also been

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

shown that IT delivery of BCG enhances protection, correlating with increased T<sub>RM</sub> numbers (17). Our study tested the hypothesis that heterologous route prime-boost vaccination strategies would enhance vaccine efficacy and T<sub>RM</sub> numbers.

As previously demonstrated, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocyte numbers in the airways and lungs increased following IT vaccination, an increase not observed following standard parenteral vaccination. The statistically significant rise in T<sub>RM</sub> numbers following IT administration of BCG::RD1 in airway and lung tissue appears to reduce

within a 20-day period (40 days after vaccination compared with 60 days after vaccination). A similar decline in lung T<sub>RM</sub> has been previously reported in other infection models, suggesting that low retention of immunocytes is an intrinsic feature of lung tissue (25, 31). On the contrary,  $CD4^+ T_{RM}$  was found 12 months after SC BCG vaccination with no measurable reduction in cell numbers in a murine model (32). However, it must be acknowledged that Bull et al. used intravascular staining and focused on lymphocytes found in the parenchyma rather than interstitium. In the present study, we did not use intravascular staining as it was logistically not feasible given the large numbers of experimental groups involved in our study. IT vaccination with BCG::pYUB only significantly increased  $T_{RM}$  numbers in lung tissue but not in the airway. Whether  $T_{RM}$ frequency in the airway or the lung tissue is more relevant to ultimate protective efficacy is currently unknown and requires further investigation. This also raises the question whether T<sub>RM</sub> induction is influenced by characteristics of the vaccine strain. In addition to the genetic modification of BCG, there has been renewed interest in BCG vaccinations administered through other routes such as intravenous, oral, and skin scarification (33). Whether inclusion of these routes of administration in prime-boost strategies can provide better protection while remaining safe is worth exploring.

It was beyond the scope of this study to determine which strategy mediated best protection at later time points. However, it is widely acknowledged that mucosal vaccination provides better protection against pulmonary TB in multiple animal models. In murine models, this differential protection is particularly evident at 45 days after infection and up until at least 100 days after infection (17). Since mucosal booster strategies were unable to provide this enhanced protection at an earlier time point and caused adverse health outcomes, it may not be worth investigating the long-term protection mediated by these strategies, unless there are avenues to improve the safety of these strategies. The exact mechanisms of why animals vaccinated through SCIT and ITIT routes suffered weight loss and clinical illness are not clear, especially since these observations did not correlate with other parameters included in our study (although these additional parameters were only investigated after illness had subsided). While a single mucosal vaccination did not cause any adverse outcomes in our study, mucosal administration of BCG in macaques resulted in a slight (<3%) reduction in body weight 3 to 4 weeks after vaccination (34). Repeated exposure of Mtb was found to cause greater lung pathology than a single exposure to the same CFU dose in a rabbit model (35). Perhaps the prime-boost strategies used in our study mimic repeated exposure to mycobacteria, inducing pulmonary damage, weight loss, and overall health deterioration. Dosing regimens used in the present study were guided by previous studies. Prime and booster vaccinations were spaced 20 days apart. as the BCG-induced systemic T lymphocyte response takes 2 to 3 weeks to reach a substantial threshold. However, it is possible that the doses used were too high or too tightly administered in time. While IT vaccination is not applicable to a clinical setting, it was used in this study under a controlled experimental setting to elicit the mucosal immune response that is likely to occur in an aerosol delivery. Aerosol delivery was not used in this study as the number of bacilli delivered is highly variable, a separate problem for translatability of live aerosol vaccines. Although modifying dose and/or prime and booster timing may result in safer vaccination strategies, in clinical settings and human challenge models, it will be almost impossible to determine when natural exposure may have last occurred.

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

Aside from assessing prime-boost BCG vaccination strategies, we also investigated ESAT-6's role in generating mycobacteria-specific T<sub>RM</sub> and immunity. ESAT-6 is known to be a major T lymphocyte immunogen during Mtb infection, and lymphocyte restimulation with ESAT-6 is used as a diagnostic test for TB. In this study, we made use of an rBCG strain (BCG::RD1 ESAT-6 Δ92-95) designed to express ESX-1- specific virulence factors, including a C-terminal truncated ESAT-6. Specific mutations of ESAT-6 have been demonstrated to attenuate virulent mycobacterial species, potentially making them more suitable for use as vaccine strains (36). A previous study with BCG::RD1 ESAT-6 Δ92-95, however, has demonstrated that it is less capable of protecting against Mtb infection than strains that express unaltered ESAT-6 (11). Animals vaccinated with BCG:: RD1 in our study produced a robust T lymphocyte response (including  $T_{RM}$ ), with a significant proportion being specific to QQWNFAGIEAAASA, an MHC-II ESAT-6 tetramer. Tetramer<sup>+</sup> cells only accounted for a small proportion of the overall increase in T<sub>RM</sub> numbers compared to BCG::pYUB- and BCG::RD1 ESAT-6 Δ92-95-vaccinated groups. This suggests that memory lymphocytes were also generated against other ESAT-6 epitopes and epitopes from other ESX-1 proteins. Although BCG::RD1 ESAT-6 Δ92-95 expresses the same repertoire of Mtb-specific antigens as BCG::RD1, it did not show the same capacity to stimulate an enhanced T cell response, instead generating memory cell numbers comparable to BCG lacking ESX-1 altogether. These data suggest that functional ESAT-6 is not only required for the noncognate production of IFN-y in vivo but is also required for the generation of a robust T lymphocyte (including T<sub>RM</sub>) response against ESX-1-secreted effectors. These results may guide the development of future rBCG strains that have the capacity to express Mtb antigens.

To account for vector-related effects on in vivo persistence and fitness, we used BCG::pYUB rather than "standard" BCG. Unexpectedly, this empty vector BCG strain induced the greatest amount of inflammation across all vaccine strategies that involved IT vaccination. Although our statistical analyses and VEIM use pYUB as the control to subtract vector effects, this highlights the importance of determining vector-related effects in early preclinical studies. However, a limitation of this study is that this strain may have caused immunopathology in excess of standard BCG, leading to an overestimation of the relative safety of the recombinant strains. Nonetheless, the VEIM, which allows objective, simultaneous evaluation and ranking of various TB vaccination strategies used by other researchers in the field, is not affected by the use of BCG::pYUB. Furthermore, our findings that SC vaccination followed by an IT boost may be unsafe and that immunogenicity does not predict efficacy can be seen in all three BCG strains used and are, hence, not attributed to the use of BCG::pYUB.

The major benefit of using live vaccines is the stimulation of immune responses that most closely resemble those of natural infection. However, live vaccines are often contraindicated in immuno-compromised individuals (those who need protection the most). Ideally, live vaccines should stimulate a strong memory response and be readily cleared. BCG has been shown to persist for up to 16 months after vaccination, allowing for a period of ongoing antigenic stimulation (*37*) but failing to confer a strong memory response in the pulmonary tissue. In our study, the numbers of recovered bacilli from the lungs of animals vaccinated with a single mucosal dose of either BCG::PYUB or BCG::RD1 ESAT-6 Δ92–95 approximately halved within a 20-day period, suggesting that these strains are being cleared from the lungs tissue. Bacilli recovered from the lungs

## SCIENCE ADVANCES | RESEARCH ARTICLE

of BCG::RD1 IT–vaccinated animals, on the other hand, remained stable at 40 and 60 days after vaccination, supporting previous studies that this strain can persist in vivo (38). Although BCG::RD1 has been deemed too virulent for human use, our data suggest that a single mucosal or parenteral dose is safe (no loss in weight or health deterioration following vaccination) in immunocompetent animals, despite its persistence. Given that antigenic stimulation appears to be necessary for the retention of pulmonary  $T_{RM}$  and only BCG::RD1 was capable of eliciting a significant *Mtb*-specific T lymphocyte response in the pulmonary tissue, our study supports a model whereby the more "virulent" strains of attenuated mycobacteria need to be considered for use as vaccines against TB (38), if a benefit in protection can be demonstrated.

Currently, there is a lack of methods that permit simultaneous assessment of our experimental readouts of protection, immunogenicity, and safety mentioned above. Thus, we have developed VEIM to integrate routinely collected data from animal studies to rank and intuitively visualize vaccine strategies based on overall performance. Using this approach, two doses of BCG::RD1 administered parenterally rank as the most favorable vaccine strategy, characterized by being equally safe (if not safer) than control BCG::pYUB, immunogenic, and appearing more efficacious in protecting against disease. This supports a growing pool of evidence that live recombinant TB vaccines should express key Mtb virulence factors to enhance protection against pulmonary TB and that mucosal booster vaccination may cause adverse outcomes if administered at the peak of the primary immune response. Furthermore, our model demonstrates that despite selecting immunogenicity parameters based on current evidence, there does not appear to be a correlation between vaccine efficacy and immunogenicity. We find a small negative correlation  $(R^2 = 0.15)$  driven by the low-immunogenic and high protection scores of BCG::RD1 (SC- or -SC) vaccination strategies. Together with previous reports (39), this finding highlights the limited understanding of the protective immune response against Mtb and correlates of protection in infection models.

Our model, to the best of our knowledge, is the first that attempts to simultaneously compare different vaccine strategies to aid the stage-gating pipeline of TB vaccine development. However, our model makes a number of assumptions, built on a priori biological premise, such as that immunogenicity, safety, and efficacy are additive effects, that different cytokines should have equal effect and are reliant on baseline control experiments to score improvements. Figure S3 shows that VEIM can be used in a versatile manner to include/exclude or to change the weighting of particular parameters in the model. This will be important to reach a consensus in the research community on how safety, efficacy, and immunogenicity should be weighted. Our model also does not consider genetic diversity, which is known to affect vaccine immunogenicity and efficacy. Thus, it would be beneficial to include additional parameters that account for genetic diversity by including studies from, for example, genetically diverse collaborative cross mice (40). Furthermore, it may be beneficial to include a wide range of parameters such as tertiary lymphoid structure generation, local antibody levels, mucosal-associated invariant T cells, and readouts of trained immunity (41, 42) in future immunogenicity studies. Nonetheless, VEIM presents as an intuitive and readily extensible approach that allows for weighting of different parameters, numbers of replicates, and pairing of samples, and a simple assessment of multiple parameters that can be extended and applied more broadly to other vaccine/infection models. In particular,

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

VEIM will be useful to dissect the relative importance of each included parameter within a vaccination challenge experiment. For example, the current gold standard for assessing the efficacy of TB vaccines in mouse models is the bacterial burden after *Mtb* challenge. However, as shown here, most current TB vaccine candidates only lead to relatively small CFU changes within a relatively narrow range. The comprehensive approach of the VEIM allows for these minor differences to be incorporated into an additive score, which allows unbiased ranking of each vaccination strategy.

The adaptability of VEIM will allow for constructive discussion within the TB research community to further fine-tune VEIM based on current evidence. The integration and visualization of multiple parameters have reinforced the need to further investigate the relationship between efficacy, immunogenicity, and safety. For example, what can we learn from the two relatively safe vaccination strategies of BCG::RD1(SC-) and BCG::pYUB (ITIT) that are highly protective, yet weakly immunogenic, or weakly protective, yet highly immunogenic to maximize overall vaccine performance?

In summary, our study provides evidence that parenteral administration of rBCG that expresses ESX-1 *Mtb* antigens enhances efficacy in protecting against TB while also maintaining a safety level comparable to control BCG::pYUB. Our study highlights potential concerns regarding the safety of mucosally administering live BCG vaccines to individuals who have previously been vaccinated, which should be considered in clinical studies and trials. We also developed a model, VEIM, which allows for evaluation of novel TB vaccine strategies to identify those that are most likely to confer better protection against TB while also being safe for human use. Approaches such as VEIM, which aim to standardize and integrate the analysis of new TB vaccine strategies and aim to identify those most worthy of further investigation, are invaluable tools for benchmarking performance against the current standard BCG delivery.

#### MATERIALS AND METHODS Animals

Female C57BL/6 mice aged 6 to 8 weeks were bred and maintained under stringent specific pathogen–free conditions in the biosafety levels 2 (BCG vaccination experiments) and 3 (*Mtb* challenge experiments) animal facilities at the Australian Institute of Tropical Health and Medicine at James Cook University, Australia. Housing conditions included regulated ambient temperature (22°C) and lighting (12-hour light/12-hour dark cycle) with unlimited access to pelleted food and water in accordance with the Australian animal rights and regulations standards (equivalent to Institutional Animal Care and Use Committee guidelines). Animals were randomly allocated to experimental groups.

#### Bacteria

BCG strains on the Pasteur background (provided by R. Brosch, Institut Pasteur, Paris) and *Mtb* H37Rv (sourced from the American Type Culture Collection) were cultured in Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.2% glycerol, 0.05% Tween 80, 10% ADC enrichment (BD Biosciences), and relevant antibiotics. Mid-logarithmic [OD<sub>600</sub> (optical density at 600 nm), 0.6 to 0.8] cultures were harvested, washed in PBS, and stored at  $-80^{\circ}$ C until required. Before vaccine administration, frozen stock vials of vaccines were thawed, and the bacteria were centrifuged for 12 min at 3500 rpm. The resulting bacterial pellet was resuspended and diluted

2020

## SCIENCE ADVANCES | RESEARCH ARTICLE

in PBS to the appropriate density as required for vaccination or infection (see below).

## Vaccinations and infection

C57BL/6 mice were immunized with commonly used doses of BCG:  $2 \times 10^5$  CFU for IT vaccination or with  $1 \times 10^6$  CFU for SC vaccination as shown in Fig. 1. Before IT immunizations, mice were anesthetized using a 1:16:160 xylazine-ketamine-PBS mixture administered intraperitoneally. Once anesthetized, mice were suspended from their upper incisors, and 20 µl of bacteria was inoculated into the oropharynx. The nostrils were occluded until the inoculum disappeared from the oropharynx. SC injections were performed into the base of the tail. These procedures were performed on day 0 (prime) and day 20 (boost) of the experimental timeline (Fig. 1). To determine protection conferred by vaccines, mice were challenged with an ultralow to low dose of Mtb H37Rv (5 to 80 CFU) via the aerosol route 90 days after vaccination using a Glas-Col inhalation exposure system. To determine the initial aerosol challenge dose, the lungs of five control mice were homogenized in 1 ml of PBS 0.05% (v/v) Tween 80, 24 hours after infection, and the entire volume of homogenized tissue was plated, incubated, and CFU was enumerated (refer to CFU enumeration below).

#### Animal weight and health score

Animals from each group were weighed weekly using an electronic scale and were assigned a daily clinical health score based on several parameters including general appearance, behavior, respiration characteristics, and response to stimuli. Full health score criteria are provided in table S1. The lowest health score assigned during the course of the week was considered the overall health score for that 7-day period.

### Sample collection

Mice were euthanized using carbon dioxide asphyxiation or cervical dislocation. Blood for serum analysis was collected in serum separator tubes (BD Biosciences) from euthanized animals through cardiac puncture and allowed to sit for 4 hours. Coagulated blood was then centrifuged at 10,000 rpm for 6 min. Sera were stored at  $-20^{\circ}$ C until analysis. To harvest the BALF, a small incision was made below the exposed larynx. Using an 18-gauge blunted needle, 1 ml of PBS was flushed into the lungs via the incision and aspirated. This was repeated three times to obtain a total of 3 ml of BALF per mouse. Organs were perfused with PBS through the left ventricle of the heart, and the lungs and spleen were harvested aseptically.

### **CFU enumeration**

For enumeration of BCG colonies after vaccination, perfused postcaval and inferior right lobes of the lung and spleen were homogenized in 1 ml of PBS 0.05% (v/v) Tween 80, and 100 µl was plated onto 10% OADC (BD Biosciences)–enriched Middlebrook 7H11 (BD Biosciences or Cell Biosciences) agar plates containing hygromycin B (50 µg/ml; Sigma-Aldrich). All strains of rBCG used were resistant to hygromycin B. For *Mtb* CFU enumeration, perfused right lungs were homogenized in 1 ml of PBS 0.05% (v/v) Tween 80, and 10-fold serial dilutions were performed (N, N<sup>-1</sup>, and N<sup>-2</sup>). Aliquots (100 µl) were plated onto 10% OADC–enriched Middlebrook 7H11 agar plates containing ampicillin (20 µg/ml), cycloheximide (50 µg/ml), 2-Thiophenecarboxylic acid hydride (2 µg/ml) (Sigma-Aldrich).

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

thus allowing for enumeration of Mtb colonies only. In both instances, plates were sealed with parafilm, wrapped in aluminum foil, and aerobically incubated for 4 weeks at 37°C. Colonies were then counted, and dilution factors were accounted for in calculating the total bacterial burden of organs.

### Lung histology

Perfused left lung lobes from vaccinated unchallenged mice and unperfused left lung lobes from challenged mice were harvested and fixed in 10% neutral-buffered formalin for 24 hours and then stored in 80% ethanol at 4°C. Tissues were then processed and embedded in paraffin (HistoCore PEARL, Leica). Sections (4  $\mu$ m) were cut and transferred onto glass slides (Snowcoat Clipped Corner Slides, Leica). The tissues were then deparaffinized using xylene and ethanol washes, rehydrated, and stained with Harris H&E using a Leica ST4020 Small Linear Stainer. Images were captured on a dissecting microscope at ×20 magnification. Histopathology/morphometric quantitation was performed using ImageJ (Fiji). The total area of lung tissue and areas of inflammatory change were calculated using the "Freehand selection" tool in ImageJ, which allows manual selection of areas of interest within an image.

#### Cell isolation and preparation for flow cytometry

Airway luminal cells were harvested from BALF. Perfused right superior and middle lobes of the lungs were used for flow cytometry sample preparation. Lung-associated cells were prepared by cutting the tissue into small pieces using scissors. The diced tissue was incubated for 30 min at 37°C in RPMI 1640 medium with 10% fetal bovine serum (FBS), collagenase VIII (0.175 mg/ml), and collagenase D (0.075 mg/ml) (Sigma-Aldrich). Single-cell suspensions were prepared using mechanical dissociated through a 70-µm nylon mesh. To eliminate red blood cells, cell suspensions were incubated with EDTA (Sigma-Aldrich) for 5 min at room temperature, washed with fluorescence-activated cell sorting (FACS) buffer solution (PBS with 2% FBS), and placed on ice until analysis.

## Antibodies, tetramer, and flow cytometry

Identification of T cells was performed by using antibodies against CD3-A700 (clone 500 A2), NKp46-BV711 (clone 29A1.4), CD4-BUV395 (clone GK1.5), CD8-BV510 (clone), CD44-BV421 (clone IM7), CD62L-phycoerythrin (PE)-Cy7 (clone MEL-14), CD103allophycocyanin (APC) (clone M290), and CD69-PE-CF594 (clone H1.2F3), all purchased from BD Biosciences. The I-A(b) Mtb ESAT-6 4-17 QQWNFAGIEAAASA tetramer (PE) was provided by the National Institutes of Health Tetramer Core Facility, USA. Fixable Viability Stain 780 (BD Biosciences) was used to exclude dead cells. Cells were incubated with viability stain for 10 min at room temperature and washed with FACS buffer solution. Samples were incubated with the tetramer (1:50 dilution) for 1 hour at room temperature and washed with FACS buffer solution. Cells were then incubated for 30 min with antibodies (1:200 dilution) on ice and washed with FACS buffer solution. Samples were resuspended in 150 µl of blank calibration particles (BD Biosciences) diluted in FACS buffer solution (1:74). Samples were analyzed using a FortessaX20 analyzer (BD Biosciences). Cells were enumerated using calibration particles 6.0 to 0.4 µm (BD Biosciences). CD3<sup>+</sup>NKp46<sup>-</sup> cells were considered T lymphocytes. Memory cells were phenotyped as follows: CD44<sup>hi</sup>CD62L<sup>hi</sup> (T<sub>CM</sub>), CD44<sup>hi</sup>CD62L<sup>lo</sup>CD103<sup>-</sup>CD69<sup>-</sup>/CD44<sup>hi</sup>CD62L<sup>lo</sup>CD103<sup>-</sup>CD69<sup>+</sup>/CD44<sup>hi</sup> CD62L<sup>lo</sup>CD103<sup>+</sup>CD69<sup>-</sup> (T<sub>EM</sub>), and CD44<sup>hi</sup>CD62L<sup>lo</sup>CD103<sup>+</sup>CD69<sup>+</sup> (T<sub>RM</sub>).

The gating strategy for identifying tetramer  $^{\rm +}$  CD4  $^{\rm +}$   $\rm T_{RM}$  is shown in fig. S2.

#### Serum multiplex analysis

Upon analysis, serum samples were thawed and prepared according to the Bio-Plex Pro Mouse Cytokine Standard 23-Plex Kit (Bio-Rad) specification. Serum samples were diluted in sample diluent (1:4), and beads and diluted samples were then incubated for 30 min at room temperature on a plate shaker at 850 rpm. The samples and beads were washed three times with wash buffer and then incubated with detection antibody as above. The samples and beads were rewashed three times and were incubated with streptavidin as previously. Following another three washes, the samples and beads were resuspended in assay buffer. Measurements were conducted on a MAGPIX instrument (Luminex).

## Serum immunoglobulin analysis

Upon analysis, serum samples were thawed and prepared according to the ProcartaPlex Mouse Antibody Isotyping Panel 2 7-Plex Kit. Serum samples were diluted in sample diluent (1:10,000), and bead and diluted samples were incubated for 60 min at room temperature on a plate shaker at 500 rpm. The samples and beads were washed. Detection antibody was added and allowed to incubate for 30 min at room temperature on a plate shaker at 500 rpm. The beads were rewashed and suspended in reading buffer. Measurements were conducted on a MAGPIX instrument (Luminex).

### Statistical analysis

Flow cytometry data were analyzed using FlowJo software version 10 (Treestar, CA). Statistical analysis was performed, and graphs were generated using GraphPad Prism version 7.0 (GraphPad software). One-way analysis of variance (ANOVA) followed by the Dunnett's multiple comparison test was used with all experimental groups being compared against unvaccinated (naïve) controls, following testing for normal distribution of data by D'Agostino-Pearson omnibus normality test. Such a statistical method was chosen so that the groups with the greatest differences were highlighted. Any bias from the use of this statistical method and the underrepresentation of statistical differences between groups were addressed through the VEIM model, which uses raw data. No data points were excluded. *P* values less than 0.05 were considered significant. Heat maps and graphs for cytokine/chemokine data were prepared on GraphPad Prism version 7.0.

## Vaccine empirical integrated model

The VEIM was implemented in R version 3.5.1. Immunogenicity<sub>x</sub> (Eq. 1), safety<sub>x</sub> (Eq. 2), and efficacy<sub>x</sub> (Eq. 3) were developed as three additive models, where x was one of the 24 vaccination strategies tested and u was the unvaccinated control. Overall scores were ranked, and the rank sum was used to rank each vaccine according to four different weighting scenarios for safety, efficacy, and immunogenicity (full results are shown in table S2 and fig. S3B). Calculations were done using the following equations

$$\begin{array}{l} \text{immunogenicity}_{x} = BALF_{x-u} + L_{x-u} + IGA_{x-u} + \\ \frac{1}{3}(IL \, 2_{x-u} + IL 12p \, 70_{x-u} + IFN_{x-u}) \end{array}$$

Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

where  $BALF_{x-u}$ ,  $L_{x-u}$ , and  $IGA_{x-u}$  represented the mean centered  $\log_{10} z$  score difference of *x* versus *u*, and cytokines  $IL2_{x-u}$ ,  $IL12p70_{x-u}$ , and  $IFN_{x-u}$  equally weighted as one cytokine score as the addition of rescaled to detection limit *z* score differences.

safety<sub>x</sub> = 
$$W_{x-u} + C_{x-u} + L_{x-u} + S_{x-u} + H_{x-u} + \frac{1}{3}(IL1 b_{x-u} + IL6_{x-u} + TNF_{x-u})$$
 (2)

where  $W_{x-u}$  represents the mean centered *z* score difference of the maximum difference of mean weight at any time point observed (weeks 1 to 6);  $C_{x-u}$  is the difference of clinical scores;  $L_{x-u}$ ,  $S_{x-u}$ , and  $H_{x-u}$  represent difference of negative rescaled to zero *z* scores for lung BCG, spleen BCG, and histopathology, respectively; and cytokines  $IL1b_{x-u}$ ,  $IL6_{x-u}$ , and  $TNF_{x-u}$  are equally weighted as one cytokine score as the addition of negative rescaled to detection limit *z* score differences

$$efficacy_x = L_{x-u} + H_{x-u}$$
(3)

where  $L_{x-u}$  and  $H_{x-u}$  represent the negative *z* score difference of lung CFUs and histopathology, respectively.

### Study approval

All animal experiments were conducted according to the Australian National Health and Medical Research Council (NHMRC) guidelines and in accordance with requirements by the animal ethics committee of James Cook University. Institutional ethics approval (A2346) for the studies was granted by the James Cook University Animal Ethics Review Committee.

#### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/10/eaaz1767/DC1

Fig. S1. Lung parenchymal immune cell profiles following vaccination. Fig. S2. FACS gating strategy to identify tetramer<sup>+</sup> CD4<sup>+</sup> T<sub>RM</sub>. Fig. S3. Vaccine empirical integrated model. Table S1. Health assessment scoring criteria. Table S2. Raw data for VEIM. View/request a protocol for this paper from *Bio-protocol*.

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13 of 14

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## SCIENCE ADVANCES | RESEARCH ARTICLE

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Muruganandah et al., Sci. Adv. 2020; 6 : eaaz1767 4 March 2020

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8.2 Appendix 2: Dysregulation of key cytokines may contribute to increased susceptibility of diabetic mice to *Mycobacterium bovis* BCG infection *https://doi.org/10.1016/j.tube.2019.02.005* 

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Dysregulation of key cytokines may contribute to increased susceptibility of diabetic mice to *Mycobacterium bovis* BCG infection



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ARTICLEINFO	A B S T R A C T
Keywords: Bacille Calmette-Guérin Murine model Macrophage Phagocytosis Type 2 diabetes Tuberculosis	Diabetes is one of the major co-morbidities contributing to the high global burden of tuberculosis (TB). The increased susceptibility of individuals with type 2 diabetes (T2D) to TB is multifactorial and may influence the efficacy of vaccines. This study was undertaken to determine the early immune responses that occur following infection with <i>Mycobacterium bovis</i> Bacille Calmette-Guérin (BCG) in a diet-induced murine model of T2D. The phagocytic capabilities of alveolar (AM) and resident peritoneal macrophages (RPM) were assessed using <i>ex vivo</i> assays. Compared to macrophages from non-diabetic mice, macrophages (RPM) were assessed using <i>ex vivo</i> susceptibility to BCG was determined following intravenous infection and diabetic mice showed a trend towards increased mortality, higher bacterial burden in the lung, liver and spleen and increased inflammatory lesions compared to controls. Differences between tissue cytokines were observed as early as one day post-infection and by days 14 and 35, lung and liver TNF- $\alpha$ and IFN- $\gamma$ levels were decreased in diabetic mice compared to controls. These results suggest that early dysregulated immune responses may influence the susceptibility of T2D mice to BCG infection.

#### 1. Introduction

The global burden of Type 2 diabetes (T2D) has increased significantly over the past 50 years [1]. T2D results in a three to four-fold increased risk of developing tuberculosis (TB) [2], other mycobacterial infections [3,4] and reactivation of latent TB (LTBI) [5]. *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) is the only TB vaccine. BCG prevents childhood TB, but the level of protection in adults varies from 0 to 80% [6,7]. Intravenous BCG vaccination has shown improved protection against TB in non-human primates [8–10]. The impact of T2D in BCG vaccination efficacy is unknown.

To explore the potential defects resulting in increased susceptibility of diabetics to mycobacterial infections and to investigate the impact of diabetes on intravenous BCG vaccination, appropriate animal models that reflect the pathophysiological mechanisms observed in T2D are crucial. The animal models that have been previously used to study

mycobacterial infection-diabetes co-morbidity were based on genetic modifications or chemical alteration of pancreatic β-cell function, which more closely model type 1 diabetes [11-18]. Furthermore. some of previously used diet-induced animal models have not been very representative of T2D due to the use of less appropriate dietary composition [19] and short dietary intervention period [20]. These are not conducive to the development of overt clinical signs of diabetes and its associated macro- and microvascular complications. To overcome some of these shortcomings we previously characterized a more robust dietinduced murine model of T2D using an energy-dense diet [3,21]. In the current study, we used BCG to investigate the antimycobacterial functions of murine diabetic versus non-diabetic macrophages, and whether diabetes influences survival and tissue bacterial burden, inflammation and cytokine level following intravenous BCG infection. We show that dysregulated tissue cytokines may render the T2D host to a greater susceptibility to mycobacterial infection.

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#### 2. Materials and methods

#### 2.1. Induction of diet-induced murine model of type 2 diabetes

Six-week-old male C57BL/6 mice were used for the induction of type 2 diabetes as previously described [3,21]. One group of mice received *ad libitum* access to an energy dense-diet (EDD), while the control group received a standard rodent diet (SRD) [21]. After 30 weeks of respective diets, glucose tolerance tests (GTT) were used to assess in sulin resistance (Supplementary Fig. 1) [21,22].

#### 2.2. Bacterial isolate and culture

*Mycobacterium bovis* BCG was kindly provided by Dr Nick West, School of Chemistry and Molecular Biosciences, The University of Queensland, Australia. The bacterial stock was grown on 7H9 medium containing 0.05% Tween 80 and supplemented with 10% OADC (Oleic Albumin Dextrose Catalase) to mid-log phase and stored at -80 °C until use.

#### 2.3. Internalization and killing assay

The capacities of murine diabetic and control alveolar macrophages (AM) and resident (non-elicited) peritoneal macrophages (RPM) to engulf and kill M. bovis BCG were evaluated using standard methods [3]. Broncho-alveolar lavage fluid (BALF) and peritoneal exudates were pooled from each group of animals (n = 30/group) prior to isolation of macrophages. CD11c<sup>+</sup> cells (AM) were isolated from BALF by positive selection using the EasySep™ Mouse CD11c positive selection kit (STEMCELL™ technologies) according to the manufacturer's instructions. CD11b<sup>+</sup> cells (RPM) were prepared from peritoneal exudate fluid using anti-CD11b Magnetic Particles-DM (BD Biosciences, Australia) according to the manufacturer's instructions. The purity of both CD11c<sup>+</sup> and CD11b<sup>+</sup> cells was assessed using the BD FACSCalibur™ flow cytometer and found to be > 90% (data not shown). The magnetically sorted cell suspensions were resuspended in RPMI 1640 (Invitrogen. Australia) culture media at a concentration of  $1 \times 10^5$  cells/ well in 1 mL in 48 well cell culture plates. Internalization, killing and cytokine production were measured at 4 h and 24 and/or 48 h, according to previously described methods [3]. Briefly, three to five replicate wells were used for each group at each timepoint. M. bovis BCG was added to wells at an MOI 1:10 and incubated at 37 °C with 5% CO<sub>2</sub>. After 4 h of co-culture, supernatants were collected for cytokine determination. The wells were treated with Amikacin (200  $\mu\text{g}/\text{mL})$  for 2 h to kill extracellular bacteria followed by washing with phosphate buffered saline (PBS, pH 7.2). To determine the killing after 24 and/or 48 h, other plates were treated and incubated as described above. All the wells were treated with Trition X-100 $^{\circ}$  (0.1%, Sigma, Australia) for 10 min and washed by centrifugation. For enumeration of colony forming unit (CFU), serial 10-fold dilutions were prepared with the cell lysates and plated on 7H11 agar plates. Mycobacterial uptake by macrophages was calculated based on the number of bacteria internalized after 4 h in comparison to bacteria added per well. Macrophage killing capability was determined by normalizing CFU at 24 and 48 h based on uptake at 4 h.

$$4 h uptake = \frac{(No. of internalized bacteria after 4 h)}{(No. of bacteria added)} X100$$

24 or 48 h survival = 
$$\frac{(No. of survived bacteria after 24/48 h)}{(No. of bacteria internalized after 4 h)}X100$$

#### 2.4. Bacterial infections

For *in vivo* infections, frozen aliquots of *M. bovis* BCG were prepared for injection as previously described [3,23]. Briefly, frozen bacterial

Tuberculosis 115 (2019) 113-120

stocks of known concentration (CFU/mL) were thawed and washed twice with PBS by centrifugation at 4000×g for 10 min. Pellets were resuspended in PBS, vortexed and clumps were disaggregated by passing the bacterial suspension through 29-gauge needles 10–15 times followed by water-bath sonication for 10 s. Serial dilutions of the resulting suspension were plated on 7H11 agar plates to retrospectively enumerate the precise CFU/mL of the inoculum. Diabetic and control mice were infected intravenously via the tail vain [3,24]. For 60 day survival experiments mice (n = 9–12/group) were infected with 2 × 10<sup>6</sup> CFU and mice were observed daily. Moribund mice were enthanized by CO<sub>2</sub> asphyxiation and survival was recorded. The kinetics of infection were assessed at days 1, 14 and 35 following infection with 1 × 10<sup>6</sup> CFU (n = 4–5/group/timepoint).

#### 2.5. Preparation of organ homogenates

At days 1, 14, and 35, 4–5 mice from each experimental group were euthanized by  $CO_2$  asphysiation for the collection of lung, liver and spleen. All samples were processed according to published protocols [3,12,25]. Briefly, lung (left lobe), liver (1 g) and spleen (half of total organ weight) were homogenized separately in stomacher bags containing 1 mL of PBS with 0.05% Tween 80. The homogenates were centrifuged, and the supernatants were collected and stored at -80 °C for cytokine assays. The cell pellets were then lysed using 0.1% Triton X-100° for 10 min. Serial 10-fold dilutions were prepared from lysates and plated on 7H11 agar plates. Colonies were counted after 2–3 weeks of incubation at 37 °C with 5% CO<sub>2</sub>.

#### 2.6. Histopathological examinations

Lung, liver and spleen of *M. bovis* BCG-infected mice were weighed at 1, 14, 35 days post-infection (dpi). For histopathological examination, the right lung lobes and liver (after removing 1 g for bacterial counts) were collected in 10% neutral buffered formalin. Tissue sections of lung and liver were prepared from formalin fixed tissue and stained with Haematoxylin and Eosin (H&E). The relative percentage of inflamed area within the lung was evaluated on representative lung sections according to previously published methods [26]. The inflamed area was calculated on the same lung sections by capturing images using 100x magnification. The inflamed areas within the liver were quantified according to previously described methods [3,27,28]. The CellSens<sup>\*</sup> Image Analysis software (Olympus) was used for digital photography and quantitative analysis of the tissue sections.

#### 2.7. Tissue Ziehl-Neelsen staining for localization of bacteria

Ziehl-Neelsen (ZN) staining of liver sections was performed at 14 and 35 dpi. Counting of acid-fast bacilli was done using previously published protocols [28]. In short, a representative liver section from each mouse was stained and visualized at 1000x magnification and the number of ZN positive magenta bacilli within each of the inflammatory focus/granuloma was counted. Inflammatory lesions showing at least one ZN positive bacillus were identified and the total number of individual bacilli within 10 such lesions were enumerated.

#### 2.8. Measurement of cytokines

Inflammatory cytokine concentrations in organ homogenates and cell culture supernatants were determined using the BD Cytometric Bead Array Mouse Inflammation Kit<sup>\*</sup> (TNF- $\alpha$ , IL-6, MCP-1, IL-10, IL-12p70, IFN- $\gamma$ ), Mouse Th1/Th2/Th17 Cytokine Kit<sup>\*</sup> (IL-2, IL-4, IL-17A) and Mouse IL-1 $\beta$  Flex Set<sup>\*</sup> (BD Bioscience, Australia). Manufacturer's instructions were followed to assess the cytokine levels. Data were acquired on a BD FACSCalibur<sup>™</sup> flow cytometer using BD CellQuest<sup>\*</sup> software (version 3.0).

## 2.9. Statistical analysis

Statistical analysis was performed using SPSS version 24.0 and GraphPad Prism 7.03 software. Normally distributed data were compared between groups using the unpaired *t*-test with Welch's correction. Normally distributed data of multiple groups were compared using the ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test. Non-normally distributed data were compared between the groups using the non-parametric Mann-Whitney *U* test. The Kruskal-Wallis test with Dunn's multiple comparisons was performed for non-normally distributed data of multiple groups. A two-way ANOVA with Sidak's multiple comparisons test was performed for data from repetitive measures (e.g. glucose tolerance test). Kaplan Meier survival curves with log-rank (Mantel-Cox) tests were used to compare diabetic and control mouse survival. All data were presented as mean  $\pm$  SEM (Standard Error of the Mean). The level of significance was indicated as \* $P \le 0.05$ , \*\* $P \le 0.01$ , \*\*\* $P \le 0.001$  and \*\*\* $P \le 0.001$ .

#### 3. Results

3.1. Phagocytosis and ex vivo cytokine production by macrophages are impaired in Mycobacterium bovis BCG-infected T2D mice

We first investigated whether the phagocytic and killing capacities of murine diabetic and non-diabetic control macrophages differed. After 4 h of co-culture, the internalization of *M. bovis* BCG by alveolar macrophages (AM) from diabetic mice was 26% lower compared to controls (Fig. 1A). Killing of *M. bovis* BCG by AM from diabetic mice after 24 h of co-culture was 4% lower compared to controls (Fig. 1B). Similarly, the bacterial uptake by resident peritoneal macrophages (RPM) from diabetic mice was 34% lower compared to control macrophages (Fig. 1D). Although the killing capacity of RPM increased over time, the killing efficiency after 24 h (Fig. 1E) and 48 h (Fig. 1G) of coculture remained 3.9 and 2.5% lower, respectively in RPM from diabetic mice compared to controls.

We also assessed cytokine levels after co-culture of isolated AM and RPM with M. bovis BCG. TNF- $\alpha$  production by AM increased between 4 and 24 h of co-culture (Fig. 1C). Compared to control mice, AM from diabetic mice produced less  $TNF-\alpha$  at both the 4 h (control. 69.76  $\pm$  0.57 vs diabetic, 44.41  $\pm$  6.14, pg/mL, P = 0.1062) and 24 h timepoints (Fig. 1C). The opposite trend was observed for IL-6 production. AM from diabetic mice released more IL-6 in response to BCG at 4 h (control, 4.00 ± 3.15 vs diabetic, 17.64 ± 2.29, pg/mL, P = 0.0096) and 24 h (Fig. 1C). The production of IL-10 by AM from diabetic and control mice was similar at both 4 h and 24 h of co-culture (data not shown). MCP-1 (Fig. 1C), IL-1 $\beta$  (Fig. 1C), IL-12p70 and IFN- $\gamma$ production was undetectable in AM from both diabetic and control mice at both timepoints. When RPM were co-cultured with M. bovis BCG for 4, 24 and 48 h, increasing TNF- $\alpha$  production was observed. TNF- $\alpha$  levels were significantly lower in RPM from diabetic mice at 4 h (control, 129.36  $\pm$  16.54 vs diabetic, 47.50  $\pm$  5.54, pg/mL, P = 0.0005) and 48 h (Fig. 1H), although concentrations were similar for both groups of mice at 24 h (Fig. 1F). MCP-1 production by RPM from diabetic mice was significantly lower than controls at 24 and 48 h (Fig. 1F and H) and was not detectable at 4 h. In contrast to our results for AM, RPM from diabetic mice produced less IL-6 than control mice at 4h (control, 601.26 ± 58.55 vs diabetic, 168.77 ± 31.78, pg/mL, *P* = 0.0001), 24 h (Fig. 1F) and 48 h (Fig. 1H) of co-culture. There was a reduced amount of IL-1 $\beta$  produced at 24 h and 48 h (Fig. 1F and H) although it was undetectable at 4 h. A higher level of IL-10 was observed in the RPM from control mice than diabetic at 48 h (control, 56.82  $\pm$  4.51 vs diabetic, 18.89  $\pm$  1.32, pg/mL,  $P \leq 0.0001)$  of coculture although the level was similar at both 4 and 24 h (data not shown). The production of IL-12p70 and IFN- $\gamma$  by the RPM from both diabetic and control mice was undetectable at all three timepoints

#### Tuberculosis 115 (2019) 113-120

 $3.2. \$  Increased trend in mortality of Mycobacterium bovis BCG-infected T2D mice

Next, we investigated if diabetic mice were more susceptible to death following high-dose ( $2 \times 10^6$  CFU/mouse) infection with *M. bovis* BCG. Sixty days after infection 30% of the diabetic mice had succumbed to infection compared to 7.69% of non-diabetic controls (Supplementary Fig. 2). Although this difference did not reach statistical significance (P = 0.1698), these data suggest that diabetic mice have a reduced ability to control BCG infection *in vivo*.

3.3. Gross pathology and increased Mycobacterium bovis BCG burden in T2D mice

To assess the immune response and histopathological changes to BCG in more detail, mice were infected with  $1 \times 10^6$  CFU and were sacrificed at 1, 14 and 35 dpi. Both diabetic and control mice developed spleno- and hepatomegaly (data not shown). Bacterial loads gradually increased in lung and liver of both diabetic and control mice followed by a decline at later timepoints (Fig. 2A and E). Diabetic mice harboured significantly more bacteria at 14 and 35 dpi compared to controls. In the lung, BCG counts peaked at 14 dpi with diabetic mice showing 2.2-fold more bacteria compared to controls and 2.9-fold more bacteria at 35 dpi (Fig. 2A). Increased bacterial burden was also observed in the liver of diabetic mice with 3.4 and 6.1-fold higher BCG counts at 14 and 35 dpi, respectively (Fig. 2E). Similarly, bacillary burden was 2.5 and 1.8-fold higher in the spleen of diabetic mice compared to controls at 14 and 35 dpi, respectively (data not shown).

#### 3.4. Increased inflammatory responses in Mycobacterium bovis BCGinfected T2D mice

Examination of H&E stained lung sections of BCG-infected mice revealed increased numbers of inflammatory lesions in diabetic mice compared to controls (Fig. 2B, C & D). A diffuse accumulation of inflammatory cells was observed in both diabetic and control groups at 1 dpi (data not shown). At 14 dpi, diabetic mice had 1.6-fold increase of inflamed area across the lung sections compared to controls (Fig. 2B). A similar trend was observed at 35 dpi (Fig. 2B, C & D).

Liver inflammation was also higher in BCG-infected diabetic mice compared to controls (Fig. 2F, G & H). At 1 dpi, a diffuse infiltration of inflammatory cells was observed in the livers of both diabetic and control mice (data not shown) followed by the formation of inflammatory foci/granulomas at later timepoints. The number of inflammatory foci/granulomas in the liver was higher in diabetic mice compared to controls at both 14 (control, 4.40  $\pm$  0.43 vs diabetic,  $6.68 \pm 0.44$ /section/mouse, P = 0.0059) and 35 dpi (control,  $6.82 \pm 0.46$  vs diabetic,  $8.08 \pm 0.78$ /section/mouse, P = 0.2010). The mean area of each inflammatory focus/granuloma was 1.2 and 1.4fold higher in diabetic mice compared to controls at 14 and 35 dpi, respectively, although this was not statistically significant (data not shown). The overall area of liver inflammation was 1.8 and 1.3-fold higher in diabetic mice compared to controls at both 14 and 35 dpi. respectively (Fig. 2F, G & H). Furthermore, ZN staining of these liver sections demonstrated higher numbers of bacilli per inflammatory focus/granuloma in diabetic mice compared to controls at both 14 (control, 13.00  $\pm$  1.08 vs diabetic, 17.56  $\pm$  3.96, P = 0.2985) and 35 dpi (control, 17.59 ± 2.09 vs diabetic, 23.25 ± 5.01, P = 0.2940) (Fig. 2I and J). Inflammatory foci/granulomas of diabetic mice appeared to be more diffuse and less compact than those of control mice (Fig. 2I and J). Moreover, compared to controls, bacilli in the liver of diabetic mice were not only localized within the inflammatory foci/ granulomas, but were also found dispersed throughout the liver parenchyma.

Tuberculosis 115 (2019) 113–120



Fig. 1. Internalization and killing of *Mycobacterium bovis* BCG by alveolar and resident peritoneal macrophages are impaired in T2D. Macrophages from both diabetic and non-diabetic (control) mice were co-cultured with *Mycobacterium bovis* BCG at an MOI 1:10 for 4 and 24/48 h. *M. bovis* BCG uptake after 4 h (A) and killing of internalized bacteria after 24 h (B) were reduced significantly in alveolar macrophages (AM) from diabetic mice compared to controls. After 24 h of co-culture with the bacterium, there was a significant reduction of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) production by AM from diabetic mice compared to controls. After 24 h (B) and IL-1 $\beta$  by AM from both diabetic and controls at the same timepoint of co-culture (C). Similar to AM, there was a significant reduction of *M. bovis* BCG uptake (D) and 24 h killing (E) by resident peritoneal macrophages (RPM) from diabetic mice compared to controls. The 48 h killing of internalized mycobacteria were also lower in RPM from diabetic mice compared to controls although no differences were found in TNF- $\alpha$  secretion by RPM from both groups of mice (F). The production of IL-6 and IL-1 $\beta$  was lower in RPM from diabetic mice compared to controls at the same timepoint of co-cultures were found in TNF- $\alpha$  secretion by RPM from diabetic mice (F). The production of IL-6 and IL-1 $\beta$  was lower in RPM from diabetic mice compared to controls at the same timepoint although these were not statistically significant (F). After 48 h of co-culture, the production of TNF- $\alpha$ , MCP-1, IL-6 and IL-1 $\beta$  were reduced significantly in RPM from diabetic mice compared to controls (H). The experiment was repeated twice with similar results. Result of a representative experiment was presented above. Data presented as mean  $\pm$  SEM; n = 3-5 replicates at each timepoint. Level of significance: \* $P \le 0.05$ , \*\* $P \le 0.01$  and \*\*\* $P \ge 0.000$ .

## 3.5. Dysregulated cytokines in Mycobacterium bovis BCG-infected T2D mice

We next assessed the levels of inflammatory cytokines in lung and liver following *M. bovis* BCG infection. At 14 dpi, TNF- $\alpha$  levels were lower in diabetic lungs compared to controls (Fig. 3A). Whereas at 14 dpi MCP-1 production was significantly higher in diabetic lungs compared to controls (Fig. 3B). No significant changes in IL-1 $\beta$  and IL-6 production were observed between the two groups (Fig. 3C and D). IFN- $\gamma$  was undetectable at 1 dpi but was significantly reduced in diabetic lungs compared to controls at both 14 and 35 dpi (Fig. 3E). In the liver similar trends were observed with TNF-α (Fig. 3F), and IFN- $\gamma$  (Fig. 3J) being significantly lower in diabetic mice compared to controls at both 14 and 35 dpi and IL-6 (Fig. 3I) significantly lower in the same group at 35 dpi. IL-1 $\beta$  production was significantly higher in liver of diabetic mice although it was not significant for 14 and 35 dpi in the same group (Fig. 3H). IL-4 levels in diabetic livers were significantly higher compared to controls at 1 (control, 2.28 ± 1.53 *vs* diabetic, 7.85 ± 0.89, pg/mL, *P* = 0.0198) and 14 dpi (control, 2.29 ± 1.82 *vs* diabetic, 14.96 ± 4.45, pg/mL, *P* = 0.0300). The production of splenic MCP-1



#### Tuberculosis 115 (2019) 113-120

Fig. 2. Type 2 diabetes increases susceptibility to Mycobacterium bovis BCG infection. Diabetic and non-diabetic (con-trol) mice were infected intravenously with Mycobacterium bovis BCG  $(1 \times 10^6 \text{ CFU}/$ mouse) and observed for a period of 35 days. Mice were sacrificed at 1, 14 and 35 days post-infection (dpi) and organ mycobacterial load and histopathological lesions were assessed. A significantly higher bac-terial burden was observed in lung of diabetic mice compared to controls at 14 and 35 dpi (A). Histological examination and quantification of the inflammatory lesions indicated a higher inflamed area in lung of diabetic mice compared to controls (B, C and D). Similar to lung, significantly higher bacterial load (E) and inflammatory lesions (F, G and H) were observed in liver of diabetic mice compared to controls at 14 and 35 dpi. Ziehl-Neelsen staining of the same liver sections demonstrated higher numbers of *M. bovis* BCG in each inflammatory focus/ granuloma of liver of diabetic mice compared to controls (I and J). Data presented as mean  $\pm$  SEM; n = 4–5 mice/group at each timepoint. Figure C, D, G and J were the representative figures of 35 dpi time-point. Magnification: C-D 40x (scale bar 500  $\mu$ m), G-H 200x (scale bar 100  $\mu$ m) and I-J 1000x (scale bar 20  $\mu$ m). Level of significance: \**P* ≤ 0.05, \*\**P* ≤ 0.01 and \*\*\* $P \leq 0.001$ .

117



Fig. 3. Cytokine production is dysregulated in Mycobacterium bovis BCG infection in T2D. Mice were infected intravenously with Mycobacterium bovis BCG (1 × 10<sup>6</sup> CFU/mouse) and sacrificed at 1, 14 and 35 days post-infection (dpi) to determine cytokine production in the lung and liver. There was a significant reduction in the production of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) at 14 dpi in lungs of diabetic mice compared to non-diabetic controls (A). The production of interleukin (IL)-1 $\beta$  (C) and IL-6 (D) was not significantly higher in the lungs of diabetic mice compared to controls at 14 dpi (B). The production of interleukin (IL)-1 $\beta$  (C) and IL-6 (D) was not significantly different between the two groups. There were significantly lower levels of interferon- $\gamma$  (IFN- $\gamma$ ) in the lungs of diabetic mice compared to controls at 14 and 35 dpi (E). Similar trends to the lungs in TNF- $\alpha$  (F), MCP-1 (G), IL-1 $\beta$  (H), IL-6 (1) and IFN- $\gamma$  (J) production were observed in the liver of diabetic and controls. Data presented as mean  $\pm$  SEM; n = 4–5 mice/group at each timepoint. Level of significance: \* $P \le 0.05$ , \*\* $P \le 0.01$  and \*\*\* $P \le 0.001$ .

at 35 dpi and IL-1 $\beta$  at 1 and 35 dpi were significantly higher in diabetics compared to controls (Supplementary Fig. 3). Whereas the production of IL-6 at 1 dpi, IFN- $\gamma$  at 35 dpi and IL-2 at 14 and 35 dpi were significantly lower in the spleens of diabetic mice compared to controls (Supplementary Fig. 3).

## 4. Discussion

Identifying the immune mechanisms underlying TB-T2D comorbidity is important for the development of effective therapeutic and preventive measures. Using a diet-induced animal model which reflects

the characteristics of T2D [3,21], this study investigated the anti-mycobacterial functions of mouse diabetic versus non-diabetic macrophages, and showed that diabetes influences animal survival and tissue bacterial burden, inflammation and cytokine levels following BCG infection.

Diabetic mice challenged with a high-dose of M. bovis BCG displayed a trend towards increased mortality. These results are consistent with other studies using Streptozotocin (STZ) induced diabetes followed by M. tuberculosis (Mtb) [13-15] and M. fortuitum infection [3]. In the current study, increased mortality was accompanied by a higher bacterial burden in the lung (Fig. 2A), liver (Fig. 2E) and spleen following a low-dose infection. A similar pattern of increased bacterial burden has been observed in STZ induced diabetic animals infected with Mtb [12,14–17,29,30]. The higher mycobacterial burden suggests that diabetic mice were unable to control infection efficiently. The increased bacterial burden in diabetic mouse tissue was further associated with increased inflammatory lesions in the lung (Fig. 2B, C and D) and liver (Fig. 2F, G and H). Previous studies have also reported an increased inflammation in lung [14-16] and liver [29,30] of STZ-induced diabetic animals infected with Mtb. The observed increase in bacterial burden in diabetic mice coupled with increased numbers and size of inflammatory lesions suggest a significant failure to control and confine the bacilli within the inflammatory lesions.

The increased bacterial burden and number and size of inflammatory lesions in diabetic mice, may be explained by impaired phagocytosis and/or dysregulated macrophage cytokine responses [3.17]. Macrophages are one of the first cell types to encounter mycobacteria within the lung [31,32]. Our study revealed significantly decreased uptake of M. bovis BCG by AM and RPM from diabetic mice (Fig. 1A and D). Defective uptake of Mtb by AM and M. fortuitum by both AM and RPM of diabetic mice has been described by others and us previously [3,18]. Reasons for the impaired diabetic phagocyte function may relate to reduced interactions between bacteria and phagocytes caused by altered complement protein (C3b) binding and/or receptor (CR1 and CR3) expression [33,34]. Furthermore, recent studies have demonstrated that diabetic macrophages have reduced expression of the scavenger receptor; macrophage receptors with collagenous structure (MARCO) [18] suggesting that both opsonin-dependent and -independent phagocytic process may be impaired in diabetics. The significantly reduced bacterial killing we observed for both diabetic AM (Fig. 1B) and RPM (Fig. 1E and G) may also be due to reduced secretion of antimycobacterial compounds, a delayed acidification of the phagosome and phagosome-lysosome fusion and altered cytokine secretion [31,32]. We observed reduced production of TNF- $\alpha$  by AM and RPM from diabetic mice (Fig. 1C and H). Our findings are consistent with previous studies in diabetic animals [3,29,30]. Decreased production of TNF-a by macrophages from diabetic animals was associated with lower uptake and killing of the bacilli. It is tempting to speculate that this could be due to the prominent role of TNF- $\alpha$  in the secretion of antimycobacterial compounds [35].

MCP-1 (CCL2) is a potent chemoattractant protein involved in recruiting various immune cells (including phagocytes) into infected tissue, Diabetic RPM produced less MCP-1 (Fig. 1F and H) suggesting an inability to recruit other immune cells. Vallerskog and colleagues [17] suggested a reduced level of MCP-1 as a cause of delayed recruitment of myeloid cells followed by delayed antigen presentation in Mtb infected diabetic mice. We observed higher levels of IL-6 produced by M. bovis BCG-infected diabetic AM (Fig. 1C) but lower levels in RPM (Fig. 1 F and H) compared to controls. Although there are conflicting reports on the role of IL-6 [36,37], the reduced IL-6 lead to higher bacterial survival. A higher level of Mtb growth in macrophages of IL-6 knockout mice has been observed previously [38]. Despite the lower production of IL-6 by AM, the increased production of TNF- $\alpha$  might compensate to limit bacterial growth. We also observed a reduced production of IL-1 $\beta$ by RPM from diabetic mice (Fig. 1F and H), which suggests that this cytokine is positively correlated with the secretion of TNF- $\alpha$  and IL-6

[39]. We detected a higher amount of IL-10 in RPM of control mice, although the survival of BCG inside the macrophages was low. The antiinflammatory environment and increased killing of internalized bacteria by macrophages from non-diabetic mice may be compensated by elevated secretion of other pro-inflammatory cytokines (e.g. TNF- $\alpha$ , MCP-1, IL-6, IL-1 $\beta$ ).

We observed significantly lower TNF-a production in the lung of BCG-infected diabetic mice at 14 dpi (Fig. 3A) which is consistent with our in vitro studies. In the liver, TNF-a was significantly reduced in diabetic mice at 14 and 35 dpi, and a similar trend was observed for IL-6 at 35 dpi (Fig. 3F and I). Similar findings have been reported in diabetic animal models following acute *Mtb* infections [15,29]. Lower production of these cytokines at the early stages of infection in diabetic mice failed to activate and recruit macrophages. Previous studies suggested that decreased production of cytokines in diabetic mice during mycobacterial infections leads to elevated bacterial burdens and increased inflammatory lesions [12,17]. Although MCP-1 production is crucial in mycobacterial defence, an increased production of this cytokine in diabetic mice at later timepoints failed to control bacillary loads, resulting in greater tissue inflammation [14,15,17]. We also observed low amounts of IFN- $\gamma$  from the lung, liver (Fig. 3E and J) and spleen (Supplementary Fig. 3E) of diabetic mice compared to controls. The central role of Th1 cells in the defence against Mtb has been attributed to the ability of Th1 cell-derived IFN- $\gamma$  to activate macrophages and stimulate phagocytosis, phagosome maturation, production of proinflammatory cytokines (e.g. TNF- $\gamma$ , IL-1 $\beta$ , IL-6) and antigen presentation [31,32]. A decreased level of IFN-y in response to BCG infection observed in this study (Fig. 3E and J) suggests impaired/delayed Th1 cell responses and leucocyte recruitment in diabetic mice. Previous research in hyperglycaemic mice have demonstrated that Mtb-specific IFN-y producing T cell recruited later in the regional lymph nodes with a proportionate delay in recruitment of those cells to the lung leading to delayed T cell priming [17]. Reduced production of this cytokine may be further explained by the decreased production of IL-2 (Supplementary Fig. 3F) that could further reduce, or at least, delay Th1 cell differentiation and recruitment as seen in TB defence [31,32]. We also observed minor increases in IL-4 in the liver of diabetic mice early in infection suggesting the possibility that the immune response may be skewed towards a more Th2 cell type. IL-4 may induce macrophages to take on a M2 (alternatively activated) phenotype [31,32]. This may down-regulate Th1 responses by inhibiting Th1 cell differentiation via influencing the transcription of IFN- $\gamma$  in Th1 effector cells [40]. Future studies are required to evaluate the role of this cytokine in diabetes-mycobacterial co-morbid infections.

Our work also suggests that delays or defects in the formation of inflammatory/granulomatous foci in the diabetic mice may be due to impaired macrophage activity. The observation of loosely associated cells in such foci and the diffusely spread bacilli in the liver parenchyma (Fig. 2.1) suggests a possible impairment in leucocyte recruitment in response to higher numbers of bacilli. In contrast, inflammatory foci/granulomas of non-diabetic mice were more compact containing lower numbers of bacteria (Fig. 21). In patients with TB who are immunodeficient, granulomas are larger in size, rich in activated macro-phages with fewer surrounding lymphocytes in contrast to immunocompetent patients with TB who have small, compact granulomas with more IFN- $\gamma$  producing CD4<sup>+</sup> T cells [41]. Future studies using relevant murine models should be aimed at characterizing the composition of the inflammatory foci/granulomas in the diabetic and non-diabetic mice.

In conclusion, impaired macrophage function is one of the key factors responsible for increased susceptibility to mycobacteria in diabetes, which ultimately leads to delayed Th1 cell mediated responses. In this investigation, we demonstrated that uptake, killing and cytokine production of macrophages from diabetic mice are impaired in early *M. bovis* BCG infection. These impairments lead to increased organ mycobacterial loads with defective or poor inflammatory or granulomatous

foci formation in diabetes due to impaired or defective Th1 cell responses. We consider the model we have developed and characterized in terms of early responses in T2D to mycobacterial infection is robust, and therefore appropriate to further investigate the impairment in development of protection in T2D-mycobacterial co-morbidity.

#### Conflicts of interest

There are no conflicts of interest relevant to this manuscript.

#### Ethical approval

All animal experiments were conducted following the National Health and Medical Research Council (NHMRC) guidelines and approved by the institutional Animal Ethics Committee (A2016).

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at https:// doi.org/10.1016/j.tube.2019.02.005.

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219

8.3 Appendix 3: A Systemic Review: The Role of Resident Memory T Cells in Infectious Diseases and Their Relevance for Vaccines Development https://doi.org/10.3389/fimmu.2018.01574

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## A Systematic Review: The Role of Resident Memory T Cells in Infectious Diseases and Their Relevance for Vaccine Development

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**Background:** Resident memory T cells have emerged as key players in the immune response generated against a number of pathogens. Their ability to take residence in non-lymphoid peripheral tissues allows for the rapid deployment of secondary effector responses at the site of pathogen entry. This ability to provide enhanced regional immunity has gathered much attention, with the generation of resident memory T cells being the goal of many novel vaccines.

**Objectives:** This review aimed to systematically analyze published literature investigating the role of resident memory T cells in human infectious diseases. Known effector responses mounted by these cells are summarized and key strategies that are potentially influential in the rational design of resident memory T cell inducing vaccines have also been highlighted.

**Methods:** A Boolean search was applied to Medline, SCOPUS, and Web of Science. Studies that investigated the effector response generated by resident memory T cells and/or evaluated strategies for inducing these cells were included irrespective of published date. Studies must have utilized an established technique for identifying resident memory T cells such as T cell phenotyping.

**Results:** While over 600 publications were revealed by the search, 147 articles were eligible for inclusion. The reference lists of included articles were also screened for other eligible publications. This resulted in the inclusion of publications that studied resident memory T cells in the context of over 25 human pathogens. The vast majority of studies were conducted in mouse models and demonstrated that resident memory T cells mount protective immune responses.

**Conclusion:** Although the role resident memory T cells play in providing immunity varies depending on the pathogen and anatomical location they resided in, the evidence overall suggests that these cells are vital for the timely and optimal protection against a number of infectious diseases. The induction of resident memory T cells should be further investigated and seriously considered when designing new vaccines.

Keywords: resident memory T cells, infectious diseases, vaccine development, immunity, microbiology

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1

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## INTRODUCTION

Traditionally, memory T cells have been subdivided into two broad categories: effector memory and central memory T cells ( $T_{\text{EM}}$  and  $T_{\text{CM}}$ , respectively). After the realization that some memory T cells fail to egress out of peripheral tissues back into the blood stream, it became clear that this dichotomous distinction of memory T cells did not account for the complete diversity of the memory T cell population. This led to the discovery of a third subset of memory T cell. Appropriately, dubbed "Tissue-resident memory T cells" (here after referred to as T<sub>RM</sub>), this newly defined population exhibits the unique feature of remaining localized in peripheral tissues (1). As such, these cells provide enhanced localized immunosurveillance and protection of peripheral tissues when compared to  $T_{\mbox{\scriptsize EM}}$  and  $T_{\mbox{\scriptsize CM}}.$   $T_{\mbox{\scriptsize RM}}$  have been characterized in many peripheral tissues, including skin, lungs, brain, liver, the female reproductive tract, and the gastrointestinal mucosa. Given the huge variance in their location of residence, this subset of memory cell is highly heterogeneous, phenotypically varying depending on their anatomic location and the inflammatory cues produced by their respective microenvironment. Although experimental techniques such as parabiosis can definitively distinguish T<sub>RM</sub> from circulating memory T cells, other less complex methods of identifying T<sub>RM</sub> are more frequently used. The co-expression of CD69 and CD103 is commonly used as a marker of tissue residence, although it appears not all bona fide T<sub>RM</sub> are defined by this particular phenotype. Regardless, T<sub>RM</sub> have been implicated in a wide range of physiological functions, such as providing protection against pathogens and cancerous cells, as well as in many pathological states such as autoimmune and other inflammatory diseases. The exploration of  $T_{\ensuremath{\text{RM}}}$  biology and the role they play in maintaining homeostasis has broad implications for human health. Currently, our understanding of T<sub>RM</sub> function is largely constrained within the context of infectious diseases. As of now, it appears that T<sub>RM</sub> are better adapted to providing rapid protection against pathogen invasion when compared to their circulating counter parts (2-4). Thus, vaccines of the future would ideally establish a population of protective  $T_{\mbox{\tiny RM}}$  at the portals of entry most at risk of pathogen invasion to provide immediate and effective immunity, rather than relying on the delayed recruitment of effector cells from the circulating pool of memory cells. Since parenterally administered vaccines induce minimal tissuespecific protection, current routes of administering vaccines may need to be revised (5, 6). The present review will primarily focus on the role of T<sub>RM</sub> in the immune response generated to a range of human pathogens and discuss future avenues for the development of T<sub>RM</sub>-based vaccines.

## METHODOLOGY

A systematic search of published literature was conducted. Literature was critically evaluated for evidence of the role  $T_{RM}$  play during infections and in vaccinology. A flowchart summarizing our methodology has been included (**Figure 1**). The preparation of this review was guided by the *PRISMA-P 2015 guideline* (7).



2

July 2018 | Volume 9 | Article 1574

Final searches of literature were performed on March 23, 2018 in Medline, SCOPUS and Web of Science by the first author. The Boolean search strategy used was as following ("resident memory t cell\*" OR "t resident memory cell\*" OR "tissue resident memory cell\*" OR "resident memory" OR "tissue memory"). The references of included studies were also screened for other relevant publications.

Both human and animal studies that use surface markers of residence or other established techniques such as intravascular staining and parabiosis to illustrate localization of T cells to peripheral tissues, as well as T cell phenotyping were included. Studies were also screened for their relevance to human pathogens, and thus animal infection models that are analogous to human infectious diseases were included. Studies were included irrespective of published date. Only published and accepted manuscripts of original research were included. Publications that primarily focused on  $T_{RM}$  biology (ontogeny, cellular metabolism, etc.) or non-infectious diseases such as hepatocellular

carcinoma and cervical cancer that can be caused by pathogens are briefly mentioned within the broader discussion of  $T_{\rm RM}{}^{-}$  mediated immunity.

## **RESULTS OF SEARCH**

The results of the search strategy are summarized in Figure 1.

## DATA SYNTHESIS AND ANALYSIS

The first author conducted extraction of data from relevant studies. This review has been divided into sections based on pathogen type: viruses, bacteria, parasites/helminths, and fungi (**Figure 2**). The studies included in this review contain the most relevant findings related to immune responses generated by  $T_{RM}$  against human pathogens, or make use of novel strategies for  $T_{RM}$  generation. We apologize to authors whose work could not be included in this review.



3

## THE ROLE OF T<sub>RM</sub> IN VIRAL INFECTIONS

As of present,  $T_{RM}$  immune responses are by far mostly studied in the context of viral pathogens. The following section will present findings by specific viral pathogen/viral disease.

## **HERPES SIMPLEX VIRUS (HSV)**

Herpes simplex virus causes infections that present with a varying range of symptoms. The primary clinical manifestations of HSV infection are intraepithelial vesicles. There are two antigenically distinct HSV subtypes: HSV-1 and HSV-2, causing cold sores and genital warts, respectively (8). However, both sub-types can be the etiology of either clinical disease as sexual transmission allows for spread between the two sites (9). Both viruses establish a life-long latent infection within the surrounding nervous tissue, and control of HSV infection requires effective cell-mediated immune responses to prevent reactivation. However, co-morbid illnesses, immunosuppressive drugs, UV exposure, and psychological stress can hinder immune control. A number of studies suggest that  $T_{\mbox{\tiny RM}}$  are implicated in controlling HSV-1 latency in the trigeminal ganglia (10). HSV-1 infection models primarily focus on infections of the skin and nervous tissue such as the trigeminal ganglia and the eyes (11-22). Following acute infection with HSV-1,  $\mathrm{CD8^{+}}\ \mathrm{T_{RM}}$  remain localized to the skin initially infected and are also found surrounding latently infected sensory ganglia (11). However, evidence suggests that multiple exposures to cognate antigen can substantially increase the  $T_{\mbox{\tiny RM}}$  population in not only the site of HSV infection, but also in distant skin (12). CD8<sup>+</sup> skin  $T_{RM}$  appear to resemble the antigen-presenting Langerhans cells of the skin, extending dendritic projections into the surrounding tissue, probably in an attempt to survey the local area for antigen (13). This is supported by evidence from confocal microscopy and intravital imaging (21) that suggests these T<sub>RM</sub> can travel between keratinocytes (13). However, unlike Langerhans cells, these T<sub>RM</sub> do not extend into the stratum corneum (13, 17). It also appears that skin T<sub>RM</sub> are not specifically attracted to virally infected cells, and thus migrate throughout the epidermis in a random manner (17, 21). By extension, it can be inferred that skin  $T_{RM}$  may take a considerable period of time before identifying virally affected cells. As such, it may be safe to assume that a critical mass of skin T<sub>RM</sub> is needed in order to afford timely protection. This notion is supported by the observation that protection appears to be dependent upon the local density of  $T_{\mbox{\tiny RM}}$  (17). Upon antigen recognition, skin T<sub>RM</sub> undergo a change in their morphological and motility pattern, decelerating their migratory rate and losing their dendricity (13, 17). This is probably indicative of a shift in role from immunosurveillance to effector function. Furthermore, the maintenance of HSV-1-specific  $T_{RM}$ populations appears to be independent of circulating T cells in both skin and trigeminal ganglia (14, 17). Skin  $T_{\mbox{\scriptsize RM}}$  appear to be able to sustain their numbers through local proliferation after secondary infection (17). However, after a combined corticosterone and stress-induced reduction of trigeminal ganglia CD8+ T cells (presumably  $T_{\mbox{\tiny RM}}$  ), there appeared to be

no increased proliferation of remaining T cells when compared to the homeostatic proliferation rates as indicated by BrdU incorporation (14). Analysis of HSV-1-specific skin and dorsal root ganglia T<sub>RM</sub> during acute immunity and later time points revealed that transcription of cytolytic molecules decreases with time. As such, T<sub>RM</sub>-based immunity in the long term may not be reliant on enhanced cytolytic effector functions, but rather on the localization of these cells at sites susceptible to reinfection (15). The chronic inflammatory response induced by persistent viral gene expression during latency leads to  $T_{\text{\tiny RM}}$ exhaustion in the brain ependymal region, rendering them unable to control HSV-1 infection (16). Perhaps the reason why T<sub>RM</sub> downregulate their cytolytic genes during times of homeostasis is because continuous expression may lead to exhaustion. Nevertheless, it appears that the generation of CD8+  $T_{\mbox{\tiny RM}}$  in skin and ganglia may be a viable option for protection against HSV-1 infection or reactivation. Local inflammation of the skin and mucosa alone can encourage the recruitment of  $T_{\mbox{\scriptsize EM}}$ to these peripheral sites where differentiation into the  $T_{\mbox{\tiny RM}}$ phenotype occurs. This was demonstrated using 2,4-dinitrofluorobenzene, a contact-sensitizing agent. Furthermore, the application of nonoxynol-9 (a spermicide agent) to the female genital tract enhanced protection against HSV challenge, correlating with higher numbers of CD103<sup>+</sup> T cells localizing to the epithelium (19). Hence, agents that can be applied to specific tissue and that cause a localized, general inflammatory response may be a strategy worth exploring for the generation of T<sub>RM</sub>. More specifically, however, the CXCL10/CXCR3 chemokine pathway appears to be vital in generating  $T_{\text{RM}}$ , as mice deficient in either CXCL10 or CXCR3 were unprotected against HSV-1 UV-B light-induced reactivation challenge. Furthermore, the administration of CXCL10 into deficient mice through the use of a neurotropic virus vector amplified  $T_{\mbox{\tiny RM}}$  in the trigeminal ganglia, conferring better protections against reactivation challenge (20). CXCL10 administration through the use of a rAAV8-CamKIIa-GFP-CamKIIa-CXCL10 vector showed similar results (18). Samples from human patients that were asymptomatic but seropositive for HSV-1 infection were used to determine which 467 HLA-A\*0201-restircted CD8+ T cell epitopes were immunodominant in the HSV-1-specific immune response. These asymptomatic individuals generated a high number of polyfunctional CD8+ T<sub>EM</sub> against three epitopes. HLA-A\*0201 transgenic mice were primed with these epitopes and subsequently treated with an ocular topical preparation containing rAAV8-CamKIIa-GFP-CamKIIa-CXCL10 to deliver exogenous CXCL10 chemokine. Results from UV-B reactivation challenge demonstrated that this strategy was able to reduce viral shedding in tears and recurrent herpetic ocular disease (18). This strategy may be beneficial in rationally developing novel vaccines against other diseases.

Human studies have also been conducted in HSV-2 infection. Samples from the genital tract of HSV-2-infected women contained populations of HSV-2-specific T cells with a  $T_{RM}$  phenotype (23). More interestingly, a population of CD8 $\alpha\alpha^+$   $T_{RM}$  that reside at the dermal–epidermal junction have also been described in biopsies of HSV-2-infected humans. This unique positioning suggests that these cells may be able to survey the neural tissue from
which virus travels to the skin during reactivation (24). Thus,  $T_{\mbox{\tiny RM}}$ play a role in the natural immune response against HSV-2 infection. Although it was already demonstrated that T cells could be recruited to peripheral tissues using inflammatory agents (19), the "prime and pull" vaccine strategy was first described in a HSV-2 infection model (25). In this study, the investigators explored the novel idea of parenterally immunizing mice and subsequently topically administering CXCL10 into the vagina before challenging with HSV-2. Mice that underwent the prime and pull protocol showed minimal signs of clinical disease and had a survival rate of 100%. Naive and parenterally immunized mice that did not receive the pull treatment developed clinical disease and exhibited high mortality rates. This strategy also demonstrated the capacity to prevent infection of sensory neurons (25). Further investigation of this protocol revealed that immunity was largely dependent on INF- $\gamma$  produced by CD8<sup>+</sup> T<sub>RM</sub> (26). Re-stimulation of this CD8<sup>+</sup> T<sub>RM</sub> was dependent on a population of CD301b<sup>+</sup> dendritic cells that resided in the lamina propria. In fact, depletion of CD301b<sup>+</sup> dendritic cells using a diphtheria toxin model rendered the prime and pull strategy ineffective and mice suffered high morbidity and mortality rates (26). Although a non-specific inflammatory stimulus such as nonoxynol-9 may be sufficient to pull CD8<sup>+</sup> T cells in to the female reproductive tract and subsequently convert them to  $T_{\mbox{\tiny RM}}$  , it appears that antigen presentation by CD301b<sup>+</sup> dendritic cells is needed for CD8<sup>+</sup>  $T_{RM}$ mediated immunity at this site. A different study that made use of a topical vaccine containing a human papillomavirus (HPV) vector expressing gB and gD ectodomains of HSV-2 has shown the capacity to generate  $T_{\mbox{\tiny RM}}$  in the reproductive tract, and reduce viral shedding and clinical disease (27). This study highlights the capacity of HPV vectors to induce T<sub>RM</sub> in the genital tract, a vaccine strategy that may be applicable to other sexually transmitted infections. HSV-2-specific CD8+ T<sub>RM</sub> can also be generated using a "chemical-free and biological-free" laser adjuvant, a protocol that could be explored in other infectious models (28). While the vast majority of studies have assessed the protective capabilities of CD8<sup>+</sup> T<sub>RM</sub> during HSV infection, very few studies have analyzed the role of CD4<sup>+</sup> T<sub>RM</sub> in HSV infections (21, 29). Intravaginal vaccination of mice with thymidine kinase negative HSV-2 (an attenuated form of the virus) provided full protection against challenge with wild-type HSV-2, independent of CD8+ T cells and B cells. Instead, parabiosis studies demonstrated that CD4+  $T_{\mbox{\tiny RM}}$  are required within the genital tract mucosa for immunity in this model. These CD4<sup>+</sup>  $T_{RM}$  are polyfunctional, secreting IFN- $\gamma$ , TNF- $\alpha$ , and IL-2, and resided in organized, non-tertiary immune structures called memory lymphocyte clusters (MLCs). These MLCs appear to assemble under the influence of macrophagesecreted CCL5. Upon antigen stimulation, the CD4+ T<sub>RM</sub> within the MLCs expand and secrete high levels of IFN-y. The investigators of this study also report that circulating memory T cells were "barely recruited" when MLCs were present in the mucosa. This suggests that CD4+ T<sub>RM</sub> may be capable of clearing or controlling infection even before recruiting signals are generated in a magnitude large enough to attract circulating T cells to the site of infection (29). It still remains necessary to explore whether a critical mass of CD4+ T<sub>RM</sub>-containing MLCs are needed within the genital tract to provide protection. It is likely that this profound role of CD4<sup>+</sup> T<sub>RM</sub> in mediating immunity during HSV-2 infection is due to the location of the infection (genital tract) rather than the viral factors alone. This is supported by the fact that MLCs have also been found in the genital tract of both human and mice secondary to infections caused by a bacterial pathogen [refer to *Chlamydia trachomatis* (*Ct*) section of this review]. Despite the difficulties in generating CD4<sup>+</sup> TRM following prime and pull vaccination, an ideal vaccine against HSV-2 should generate CD8<sup>+</sup> and CD4<sup>+</sup> T<sub>RM</sub> (25).

#### **INFLUENZA**

Influenza viruses are a major cause of respiratory infections. Although influenza vaccines have been in use for many years, antigenic drift of surface hemagglutinin and neuraminidase proteins require annual immunizations. Antigenic shift can result in highly virulent strains of influenza that cause devastating pandemics (30). The ideal influenza vaccine would provide heterotypic immunity that prevents the escape of newly mutated viruses. Current influenza vaccines rely on generating high neutralizing antibody titers to protect against infection. Although this strategy has demonstrated efficacy in mediating protection, the inability of antibodies to neutralize new variants of the virus has sparked research into alternate strategies (31). Growing evidence suggests that efforts should be focused on developing vaccines that generate T<sub>RM</sub>-mediated immunity (32). Analysis of human samples has revealed that influenza-specific T<sub>RM</sub> can be found in substantial numbers in lung tissue, highlighting their role in natural infection (33, 34). Despite expressing low levels of granzyme B and CD107a, these CD8<sup>+</sup>  $T_{RM}$  had a diverse T cell receptor (TCR) repertoire, high proliferative capacities, and were polyfunctional (34). Influenza infection history suggests a greater level of protection against re-infections likely due to the accumulation of CD8<sup>+</sup>  $T_{RM}$  in the lungs (35). Furthermore, the natural immune response to influenza A virus infection in a rhesus monkey model demonstrated that a large portion of influenza-specific CD8+ T cells generated in the lungs were phenotypically confirmed as CD69+CD103+ T<sub>RM</sub> (36). Unlike lung parenchymal T<sub>RM</sub>, airway CD8<sup>+</sup> T<sub>RM</sub> are poorly cytolytic and participate in early viral replication control by producing a rapid and robust IFN-y response (37, 38). Bystander CD8+ T<sub>RM</sub> may also take part in the early immune response to infection through antigen non-specific, NKG2D-mediated immunity (39). The generation of functional T<sub>RM</sub> that protect against heterosubtypic influenza infection appear to be dependent on signals from CD4+ T cells (40). A role for CD4+  $T_{\text{RM}}$  has also been reported (41). Much like their CD8<sup>+</sup> counterparts, CD4<sup>+</sup> T<sub>RM</sub> also produce a significant IFN-y response during early infection (42, 43). Aside from the CD8<sup>+</sup> and CD4<sup>+</sup> subsets of  $T_{RM}$ , a subset of NK1.1<sup>+</sup> double negative T memory cells which reside in the lungs also play a role in influenza infection (44). Taken together, these studies and others (45-47) demonstrate that T<sub>RM</sub> are required for optimal protection. However, unlike  $T_{\mbox{\tiny RM}}$  in other locations, such as the skin, lung T<sub>RM</sub> are not maintained for extended periods of time. This gradual loss of lung T<sub>RM</sub> appears to be the reason for the loss in heterotypic immunity against influenza infection (45, 46, 48). Lung T<sub>RM</sub> exhibit a transcriptional profile that renders them susceptible to apoptosis (48). Despite conflicting evidence (49), it appears that maintenance of the lung CD8+ T<sub>RM</sub> populations relies on the continual seeding from circulating CD8<sup>+</sup> T cells. However, with time, circulating CD8+ T cells adopt a transcriptional profile that reduces their capacity to differentiate into  $T_{\text{RM}}$  Expanding the CD8+  $T_{\text{EM}}$  compartment through booster vaccination may circumvent the problem of these time-sensitive transcriptional changes (48). There is also conflicting evidence regarding the requirement of local antigen for the generation of  $T_{RM}$  within the lung (48, 50). Continuing to find ways to generate and maintain lung T<sub>RM</sub> is of great importance for vaccines against pulmonary infections. Intranasal administration of vaccines seems to encourage the development of a strong mucosal immune response (51, 52). Intranasal administration of Live Attenuated Influenza Vaccine (FluMist) in a mouse model induced both CD4<sup>+</sup> and CD8<sup>+</sup>  $T_{RM}$  that provided a degree of cross-strain protection independent of  $T_{\mbox{\scriptsize CM}}$  and antibodies (53). The intranasal administration of a PamCys2 or Adjuplex has demonstrated capacity for producing protective influenzaspecific lung CD8<sup>+</sup>  $T_{RM}$  in similar numbers and IFN- $\gamma$  secreting potential when compared to the natural response to influenza infection (54, 55). Furthermore, a vaccine containing virus-like particles with tandem repeat M2e epitopes generated heterotypic immunity through the induction of antibodies, and protection correlated with IFN- $\gamma\text{-secreting CD8}^+$   $T_{\text{\tiny RM}}$  (56). A Modified Vaccinia Ankara-vectored virus expressing conserved influenza nucleoprotein and matrix protein 1 elicited an IFN-y secreting CD4+  ${\rm \hat{T}}$  cell and CD8+  $T_{RM}$  response (57). Co-administration of 4-1BBL (CD137 signal) along with an influenza nucleoprotein expressing replication defective adenovirus vector via the intranasal route stimulated and boosted a lung CD8+ T<sub>RM</sub> response through the recruitment of circulating T cells (58). Intranasal administration of 4-1BBL may serve as a promising "pull" strategy in systemically primed individuals. Another potential "pull" strategy is the intranasal administration of Fc-fused IL-7. This protocol was used as a pre-treatment before influenza A infection, and demonstrated protective capacities in mice against lethal challenge. It appears that Fc-fused IL-7 recruits polyclonal circulating T cells into the lungs, which subsequently reside in the lung tissue as "T<sub>RM</sub>-like cells" (59). Intranasal administration of Fc-fused IL-7 after systemic priming may be able to recruit influenza-specific T cells into the lungs, and may be a strategy for inducing lung T<sub>RM</sub>. An antibody targeted vaccination strategy in which antigens are coupled to monoclonal antibodies against CD103<sup>+</sup> or DNGR-1<sup>+</sup> dendritic cells has also been shown to elicit a protective CD8<sup>+</sup> T<sub>RM</sub> response (47, 60).

## HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Human immunodeficiency virus is a retrovirus that is transmitted *via* contact with infected blood and other fluids such as semen and vaginal secretions. The virus specifically targets the surface proteins CD4, CXCR4, and CCR5, with the natural progression of disease resulting in the depletion of CD4<sup>+</sup> T cells. As a consequence, infected individuals are left in an immunocompromised state referred to as aquired immunodeficieny syndrome (AIDS), which is characterized by fatal opportunistic infections and malignancies. Although the development of therapeutics such as anti-retroviral therapy has reduced the incidence of AIDS, HIV/AIDS continues to contribute significantly to global morbidity and mortality. Evidence shows that CD8<sup>+</sup> T cells are vital in controlling early infection (61). Studies of human tissue samples have revealed that  $T_{\mbox{\tiny RM}}$ are generated in response to HIV infection in multiple locations including the gastrointestinal tract and the female reproductive tract (62-65). Furthermore, individuals who appeared to naturally control infection had T<sub>RM</sub> that were capable of producing the highest polyfunctional immune responses when compared to individuals who did not. However, the  $T_{\mbox{\tiny RM}}$  population within the HIV-specific CD8+ T cell compartment in individuals who controlled infection was under-represented when compared to individuals who were viremic (62). Although not confirmed, this may be due to the higher ability of polyfunctional  $T_{\mbox{\tiny RM}}$  in these individuals to recruit circulating T cells, thereby only altering the T<sub>RM</sub> proportion. Similar to other infections in various sites, CD8+  $T_{\mbox{\tiny RM}}$  in the context of HIV can be sub-divided into two subsets based on the expression of CD103 (62, 63). Analysis of the ectocervial epithelium and menstrual blood revealed that HIV-infected women were more likely to have CD103<sup>-</sup> T<sub>RM</sub> when compared to healthy individuals (63, 64). This reduced expression of CD103 may be explained by the HIV-induced depletion of CD4+ T cells which appear to be vital in providing help to CD8+ T cells for up-regulating CD103 (64). The CD103<sup>-</sup> populations of the ectocervix resided closer to the basement membrane of the epithelium when compared to their CD103<sup>+</sup> counterparts. Interestingly, the CD103<sup>+</sup> population from infected individuals appears to express higher levels of PD-1 (63). In a separate study, adipose PD-1+ CD4+ T<sub>RM</sub>, appeared to remain relatively inactive during HIV infection and may serve as a reservoir for HIV (65). As such chronically activated T<sub>RM</sub> and T<sub>RM</sub> exposed to immunomodulated environments (such as the adipose tissue) may be unable to elicit a full effector response, favoring the progression of HIV infection. It also appears that HIV has the ability to disrupt CCR5mediated CD8+ T cell migration into the cervical mucosa, thereby impairing the development of T<sub>RM</sub> populations (66). Regardless, human studies suggest that  $T_{\text{\tiny RM}}$  , especially CD8+  $T_{\mbox{\tiny RM}\mbox{\tiny ,}}$  play an important role in combating HIV infection and thus may be valuable targets for vaccine development. Since the most common mode of transmission of HIV is through sexual intercourse, it may be desirable to explore strategies that induce anti-HIV CD8+ T<sub>RM</sub> in the female and male reproductive tract and rectosigmoid epithelium. In a Simian Immunodeficiency Virus model of rhesus macaques, intravenous administration of SIVmac239 $\Delta$ nef generated a population of CD8<sup>+</sup> T<sub>RM</sub> in the vaginal tissue and the gut that participated in protection (67). In a murine model, a mucosal vaccination strategy in which intranasal administration of an influenza-vector expressing the HIV-1 Gag protein p24 followed by an intravaginal booster induced CD8<sup>+</sup>  $T_{\text{RM}}$  in the vagina. Antigen stimulation of these CD8<sup>+</sup> T<sub>RM</sub> resulted in the recruitment of B cells, natural killer cells, and CD4+ T cells (68). While the recruitment of innate

and adaptive immune cells may be beneficial in early viral clearance, the recruitment of CD4<sup>+</sup> T cells may be detrimental in the context of HIV as they are the target for HIV. Hence, incidental recruitment of CD4+ T cells to sites of HIV entry (female reproductive tract and rectum) by prime and pull vaccination strategies may unintentionally increase susceptibility to infection. A micro-needle array delivery system that utilizes a recombinant adenovirus vector containing the HIV-1 protein Gag, has also produced promising results in generating  $T_{\text{RM}}$ . These HIV-specific T<sub>RM</sub> were found in the female reproductive tract and respiratory tract of immunized mice and responded to local antigenic stimulation through expansion and production of IFN- $\gamma$  and granzyme B (69). Using this micro-needle array delivery system as a priming strategy followed by intravaginal delivery of a booster concoction serving as a pull strategy may be an interesting protocol worth exploring.

#### VACCINIA

Vaccinia is a poxvirus that usually causes a very mild or asymptomatic infection in immunocompetent individuals. Immunity to vaccinia virus also provides sufficient protection against smallpox, which allowed for its eradication following administration of the live vaccinia virus (70). Despite elimination, smallpox remains a priority on the global agenda given the potential for the virus to be used as a biological weapon (71). For this reason and its ability to serve as a vector, vaccinia virus continues to be used in research. Murine models demonstrate that TRM are generated in response to vaccinia and play a significant role in mediating protection against infection (72–76). Dermal-resident  $\gamma\delta$  T cells have also been implicated in the immune response against cutaneous vaccinia infection (77). Following skin infection CD8+ T cells are recruited independently of CD4 T<sup>+</sup> cells and IFN-γ (72), many of which subsequently assume the  $T_{RM}$  phenotype (72, 73, 75, 78, 79) and are capable of initiating potent inflammatory responses upon re-stimulation (79). Of particular interest is the capacity of local vaccinia skin inoculation to globally seed skin tissue even at remote sites with long lasting  $T_{RM}$  (72) as well as generating T<sub>RM</sub> responses in non-related non-lymphoid organs such as the lungs and liver (76). Multiple exposures to cognate viral antigens have also shown to selectively expand  $T_{\mbox{\tiny RM}}$ (72, 73, 78, 79). In a lung infection model of vaccinia, higher numbers of lung  $T_{\text{RM}}$  correlated with better protection against subsequent infection as indicated by a rapid reduction in viral loads. T<sub>RM</sub> seem to expand more rapidly and localize to the infection site as indicated by a 5-ethynyl-2'-deoxyuridine proliferation assay when compared to their circulating counter parts. Depletion of lung CD8+ T cells by intranasal administration of αCD8 antibody, resulted in previously protected mice becoming susceptible to infection, indicating that CD8+  $T_{\text{\tiny RM}}$  play a vital role in mediating immunity (74). In another study, parabiosis experiments demonstrated that T<sub>RM</sub> were exceedingly better at clearing vaccinia virus skin infection than T<sub>CM</sub> within a shorter timeframe. In fact, it appears that skin  $T_{\mbox{\tiny RM}}$  can clear vaccinia skin infection even in the absence of neutralizing antibodies and T<sub>CM</sub> (72). However, vaccinia-specific CD8<sup>+</sup> skin T<sub>RM</sub> appear to have an impaired ability to recruit circulating effector cells T<sub>RM</sub> in Infectious Diseases and Vaccines

during polymicrobial sepsis infection (80). Whether there are other physiologically challenging conditions that impair skin T<sub>RM</sub> functionality remains largely unexplored. Surprisingly, vaccinia lung infection revealed that not all  $T_{\mbox{\tiny RM}}$  are equally capable of conferring protection.  $T_{\mbox{\tiny RM}}$  that resided in the lung interstitium were better positioned to rapidly kill infected lung cells in a contact-dependent manner when compared to  $T_{\mbox{\tiny RM}}$ situated in association with the tissue vasculature. Furthermore,  $T_{\mbox{\tiny RM}}$  found within the interstitium, unlike vascular-associated  $T_{\text{RM}}$ , were able to up-regulate CD69 expression, potentially indicating an enhanced ability to respond during early infection (74). Investigations of vaccinia infection has also reinforced that epithelial immunization routes, such as skin scarification and intranasal exposure, demonstrate significant efficacy for generating protective T<sub>RM</sub> responses (72–76, 78). In fact, vaccination via skin scarification is capable of protecting against clinical disease (pock lesions of the skin) whereas not all mice vaccinated via systemic routes such as intramuscular and intraperitoneal were protected from pock lesions. More astonishingly, mice immunized via skin scarification demonstrated greater resistance to disease when challenged via a heterologous route (intranasal), compared to mice immunized subcutaneously or intraperitoneally, in spite of generating reduced antibody titers (75). These observations may be attributed to T<sub>RM</sub>-mediated immunity given the evidence that T<sub>RM</sub> can be generated in distant tissues after skin scarification (76). Overall, studies that use vaccinia infection models have shed light on the ability of skin scarification to elicit a robust and somewhat unique immune response.

#### **RESPIRATORY SYNCYTIAL VIRUS (RSV)**

Respiratory syncytial virus is a common cause of lower respiratory tract infections in children and the elderly. Common reinfection with RSV suggests absence of protective immunity (81). A number of studies have shown the importance of  $T_{RM}$ in providing protection against RSV (82-86). An experimental human infection study showed that adults with higher frequencies of RSV-specific CD8+ T cells, many of which displayed a T<sub>RM</sub> phenotype, developed less severe lower respiratory tract symptoms and reduced viral loads. This increase in protection was not correlated with higher numbers of circulating CD8+ T cells, suggesting the localization of T<sub>RM</sub> was vital for mediating immediate protection (82). T<sub>RM</sub> induction in lung tissue and airway fluid was also demonstrated following intranasal RSV infection in mice. Adoptive transfer of airway lymphocytes from RSV-infected mice into naïve recipients reduced disease burden upon infection challenge, compared to adoptive transfer of airway lymphocytes from sham-infected mice. It was concluded that both airway CD8<sup>+</sup> and CD4<sup>+</sup> T cells play a role in protecting against RSV infection and reducing disease severity, respectively (83). However, given that only bulk CD4<sup>+</sup> or CD8<sup>+</sup> T cells were transferred, it remains to be investigated if the protective capacity is mediated by airway  $T_{\mbox{\scriptsize EM}}$  or  $T_{\mbox{\scriptsize RM}}$  cells. In support of the latter, an African green monkey model of RSV infection illustrated that antibody and CD4+ T cell responses are unlikely to protect against reinfection. On the contrary, it appears that lung CD8+ T cells, of

Frontiers in Immunology | www.frontiersin.org

July 2018 | Volume 9 | Article 1574

which up to half displayed a  $T_{\text{RM}}$  phenotype, were more capable of protecting against secondary infection (84). From the available evidence (82-86), it appears that an ideal RSV vaccine should elicit a CD8+  $T_{\text{\rm RM}}$  response in the lung. Of note, some experimental RSV vaccines have already shown promising results with regards to T<sub>RM</sub> generation: intranasal administration of an RSV antigen-expressing murine cytomegalovirus generated an IFNyand MIP-1 $\beta$ -secreting population of T<sub>RM</sub> (85); co-administration of the TLR9 agonist CpG and an inhibitor of notch signaling (L-685,458) with formalin-inactivated RSV elicited a strong protective T<sub>RM</sub> response (86); intranasal administration of virus-like particles containing RSV M and M2 proteins as antigen delivery systems has also shown propensity to induce the production of T<sub>RM</sub> (87); and a dendritic cell-Listeria monocytogenes immunization strategy, when administered locally, was able to avoid circulating T cell-induced immunopathology and protect against RSV infection challenge through the generation of  $T_{RM}$  (88).

## **CYTOMEGALOVIRUS (CMV)**

Cytomegalovirus establishes life-long latency in many organs including mucosal tissues. It has long been known that CMV infection induces a sustained clonal expansion of specific CD8+ T cells, a phenomenon referred to as memory inflation (89). However, only recently has it been explicated that CMV infection promotes the formation of T<sub>RM</sub> in various mucosal tissues, especially the salivary glands (90-92). Although the CD8<sup>+</sup> T cell response is vital for the control of CMV infection, the virusinduced downregulation of MHC I on acinar glandular cells of the salivary glands (long-term target tissue of CMV) resulting in the reliance on CD4+ T cells for control of lytic replication at this site (93). Surprisingly, salivary gland CD8<sup>+</sup> T<sub>RM</sub> were capable of controlling viral replication. It appears that murine CMV is unable to completely inhibit the expression of MHC I on CD8+ T<sub>RM</sub> of the salivary glands, thereby providing an opportunity for these T cells to mediate localized immunity (90). Although it remains unclear whether these  $T_{\mbox{\tiny RM}}$  inhibit viral replication through effector cytokines or direct cytotoxicity, it certainly appears that salivary gland T<sub>RM</sub> may inhibit the shedding of CMV, hence reducing the chances of transmission. These mucosal  $T_{\text{RM}}$ typically form early after infection. However, mucosal seeding continuously occurs through the recruitment and differentiation of circulating populations. As such, the immunodominance of mucosal  $T_{\mbox{\tiny RM}}$  against CMV changes with time, favoring the TCR repertoire that remains high in circulation (91). T<sub>RM</sub> have also been found in brain tissue after murine CMV infection (94-96). In the brain, CMV-specific  $T_{\mbox{\tiny RM}}$  formation seems to be dependent on regulatory T cell (Treg) activity. Furthermore, Treg cells seem to have a suppressive effect on brain T<sub>RM</sub>'s capacity to produce granzyme B, potentially a precautionary measure to prevent detrimental neuroinflammation (94). From the studies that have dissected the role of T<sub>RM</sub> in protecting against CMV infection and inhibiting reactivation there seems to be a clear role for these tissue tropic T cells in limiting CMV replication. A number of studies also demonstrate the capacity of CMV to be used as a viral vector in novel vaccines that generate T<sub>RM</sub>-mediated immunity (85, 91, 97). Manipulating CMV's capacity to induce a robust CD8<sup>+</sup> T cell response within mucosal tissues may be a promising avenue for the generation of new vaccines.

# LYMPHOCYTIC CHORIOMENINGITIS VIRUS (LCMV)

Lymphocytic choriomeningitis virus, a rodent-borne disease can cause meningoencephalitis in humans (98). While LCMV infection models have been used to study T<sub>RM</sub> in multiple tissues (99), the protective role of  $T_{RM}$  has only been clearly investigated in the brain, thymus, and female reproductive tract. Depletion of circulating T cells or NK cells demonstrated that  $T_{\mbox{\tiny RM}}$  have the capacity to protect against infection independently of NK cells,  $T_{CM}$ , and  $T_{EM}$  populations (100, 101). Upon MHC-I-antigen stimulation, LCMV brain  $T_{\mbox{\tiny RM}}$  displayed effector functions and mediated virus control through IFN-y release and perforinmediated cytotoxicity (100). Thymic T<sub>RM</sub>, when stimulated with gp33, released both IFN- $\gamma$  and TNF- $\alpha$ , suggesting that  $T_{\text{RM}}$  at this location may be polyfunctional. It also appears that T cells that took residence in the thymus were more likely to respond to antigen stimulation when compared to their splenic counterparts, further exemplifying the protective nature of these cells (101). Since infection of the thymus can significantly reduce T cell generation due to increased thymocyte deletion and reduced proliferation, it is vital to have protective mechanisms in place that act rapidly to minimize pathogen-induced damage in the thymus. From the available evidence, thymic  $T_{\mbox{\tiny RM}}$  seem to be capable of adequately fulfilling this task. LCMV infection also induces the production of T<sub>RM</sub> in various peripheral tissues, such as the lungs, intestines, and female reproductive tract (102–104). While the role of  $T_{RM}$  in the lung and intestines following LMCV infection is not well established, it was found that re-activation of CD8+  $T_{\mbox{\scriptsize RM}}$  in the female reproductive tract was able to produce a general anti-viral immune response that is almost able to confer sterilizing immunity when challenged with an non-cognate virus (105). This T<sub>RM</sub> induced antiviral state may be of great interest in the aim to generate vaccines that create heterotypic protection.

## VARICELLA ZOSTER VIRUS

Varicella zoster virus, the cause of chicken pox, is an alphaherpes virus that can establish latency within the dorsal root ganglia. Reactivation of the virus results in a painful disease called shingles. Although vaccines are available against both chicken pox and shingles (106), recent evidence suggests that  $T_{RM}$  may be key players in controlling latent infection, a phenomenon that could be exploited to improve current vaccines. One study analyzed skin samples from human donors of varying ages who were serologically confirmed VZV positive. 80-90% of T cells from the sampled tissue expressed CD69, suggesting that the majority of T cells in skin were  $T_{RM}$ . IL-2 responses from stimulated VZV-specific T cells demonstrated that host age did not influence the numbers of responsive cells. However, it was found that skin from older donors demonstrated a lesser capacity to mount a clinical response and

Frontiers in Immunology | www.frontiersin.org

July 2018 | Volume 9 | Article 1574

decreased CD4<sup>+</sup> T cell infiltration when challenged with VZV antigen. This correlated with higher proportions of Foxp3<sup>+</sup> cells. Furthermore,  $T_{RM}$  of older skin expressed PD-1 in higher amounts (107). Together, this data suggest that VZV-specific  $T_{RM}$  may be suppressed with age. This may be a reason for the high incidence of reactivation of VZV in older individuals. Results from a different study that utilized samples of human trigeminal ganglia suggests that  $T_{RM}$  do not seem to play a role in controlling latent infection in the trigeminal ganglia (10). Regardless, further investigation into the role of  $T_{RM}$  in controlling latent VZV infection may help to develop therapeutics or vaccines that prevent shingles.

## HUMAN PAPILLOMAVIRUS

Human papillomavirus is a sexually transmitted pathogen that generally causes an asymptomatic, self-limiting infection. However, certain subtypes of HPV can cause cancer of the cervix, anus, and oropharynx (108). The routine administration of HPV preventative vaccines has led to a significant reduction in the incidence of infection in many parts of the world. However, immunization of individuals with an established HPV infection has not shown to protect against the progression of HPV-induced lesions into carcinoma. As such, a therapeutic vaccine that is administered by post infection may subvert this problem. Current HPV vaccines rely on the induction of antibodies to neutralize viral particles (109). The potential for generating anti-HPV T<sub>RM</sub> as a strategy for eliminating previously established HPV infection is yet to be fully explored. One study evaluated the capacity of two adenoviruses (Ad26 and Ad35) that express a fusion of the HPV16 oncoproteins E6 and E7 to elicit a protective response in the cervicovaginal mucosa. Intra-vaginal administration of either vector was able to elicit the generation of CD8+  $T_{\text{RM}}$  within the cervicovaginal mucosa. Furthermore, systemic priming with Ad35 followed by an intra-vaginal booster immunization of Ad26 induced polyfunctional, E6/E7-specific, cytokine-secreting CD8+ T cells within the cervicovaginal mucosa (110, 111). Although it remains to be resolved if protection against established HPV infection causally relies on T<sub>RM</sub>, this and other studies (111) provide impetus to further explore the intra-vaginal route of administration and the use of viral vectors as strategies for the induction of cervicovaginal  $T_{\text{RM}}$ .

## **VIRAL HEPATITIS**

Viral hepatitis is an inflammatory disease of the liver that is caused by a range of viruses (112). Two studies, both of which utilized human donor liver tissue and paired blood samples, analyzed the role of  $T_{\rm RM}$  in the context of viral hepatitis. One study focused on patients with hepatitis B viral infections (HBV), while the other study included patients with HBV or hepatitis C viral infections. A higher proportion of liver T cells from patients who demonstrated partial control of HBV infection had a  $T_{\rm RM}$  phenotype, when compared to healthy controls. Given that the overall numbers of T cells in the liver of healthy and HBV-infected individuals were similar, this threefold increase in  $T_{\rm RM}$  numbers appear to be due to an increased

predisposition of T cells to adopt the T<sub>RM</sub> phenotype in virally infected liver tissue, rather than expansion of pre-existing  $T_{RM}$ (113). The numbers of T cells co-expressing CD69 and CD103 increased by fourfold in chronic hepatitis C patients (114). Furthermore, the reciprocal relationship between viral loads and liver  $T_{\text{RM}}$  numbers indicates that  $T_{\text{RM}}$  play a vital role in infection control (113). Ex vivo stimulation of T<sub>RM</sub> showed heterogeneous antigen specificity, with a number of HBV antigens being able to initiate effector responses. However, viral envelope peptides seemed to generate the greatest capacity to induce production of IFN $\gamma,$  TNF $\alpha,$  and IL-2. Analysis of  $T_{\text{RM}}$ from healthy liver tissue revealed a noticeably reduced expression of granzyme B, when compared to non-resident counter parts. This suggests that hepatic T<sub>RM</sub> have less cytolytic capacity than circulating T cells (113, 114). However, liver T<sub>RM</sub> of patients with chronic hepatitis B expressed markedly higher amounts of granzyme B when compared to healthy controls (114). Liver  $T_{RM}$ also showed increased expression of the inhibitory molecule PD-1 compared to non-resident T memory cells (113, 114). The downregulation of granzyme B and upregulation of PD-1 in healthy liver tissue may be a precautionary measure intended to prevent immunopathology, given the liver's role in filtering high amounts of antigen draining from the mesenteric circulation. This is of great importance in viral hepatitis infections as immunopathology is largely involved in the progression of viral hepatitis that leads to cirrhosis and hepatocellular cancer. The increased production of granzyme by T<sub>RM</sub> in CHB patients may be part of the pathogenesis of fulminant hepatitis. Further exploring the role of  $T_{\mbox{\tiny RM}}$  in protection against viral hepatitis (including hepatitis A, D, and E) and the immunopathology implicated in the progression of the disease may aid in the development of immunomodulatory therapeutics to prevent viral cirrhosis and hepatocellular cancer.

# **EPSTEIN-BARR VIRUS (EBV)**

Epstein-Barr virus is one of the most prominent causes of infectious mononucleosis. After exposure to infected saliva, the virus infects and replicates in B cells and epithelial cells of the new host. Although the clinical disease of glandular fever is usually selflimiting, EBV remains latent in circulating B cells and episodes of reactivation are known to occur. It appears that reactivation of EBV occurs in the lymphoid tissue of the oropharynx, where the virus switches from a latent form into a lytic cycle. Control of infection is mediated by a T cell response against infected B cells (115). EBV-specific CD8+ memory T cells localize to the epithelium of the oropharynx (116), where they up-regulate CD69 and CD103 in an IL-15- and TGF- $\beta$ -dependent fashion (117). CD103<sup>+</sup> EBV-specific T memory cells found in tonsillar tissue are more sensitive to antigen stimulation and produce a greater effector response when compared to circulating EBVspecific T cells (116). Furthermore, a substantial CD103<sup>+</sup> T cell population only seems to appear as viral replication and disease tapers (118). Taken together, it appears as though  $T_{\mbox{\tiny RM}}$  play a crucial role in rapidly controlling viral replication of EBV within the oropharyngeal tissue upon reactivation to prevent full clinical relapse.

July 2018 | Volume 9 | Article 1574

Frontiers in Immunology | www.frontiersin.org

#### VESICULAR STOMATITIS INFECTION (VSV)

Vesicular stomatitis infection is a zoonotic disease that can cause a mild febrile illness in humans (119). Intranasal infection of mice with VSV has shown to produce CD103<sup>+</sup> CD8 T<sub>RM</sub> population in the brain (120, 121), as the virus travels along the olfactory bulb to the brain where it causes infection. These brain  $T_{\mbox{\tiny RM}}$  were found to be functional in situ, responding to cognate antigen (120, 121). Staining for effector molecules revealed that many of these  $T_{\text{RM}}$  cells were positive for granzyme B, suggesting cytolytic abilities. Once removed from the brain parenchyma, these cells appear dysfunctional, suggesting they are highly adapted to the brain microenvironment. Maintenance of this population of T<sub>RM</sub> appears to be independent of circulating T cells, and BrdU incorporation indicates a slow homeostatic rate of proliferation to sustain the population (120). Interestingly, brain T<sub>RM</sub> appear to form clusters within specific sites of the brain parenchyma that contain CD4<sup>+</sup> T cells, perhaps indicating a role for CD4<sup>+</sup> T cells in the generation and/or maintenance of brain CD8<sup>+</sup> T<sub>RM</sub>. These clusters may have formed around sites of previous VSV replication sites, where persisting antigen may be drawing the  $T_{\mbox{\tiny RM}}$ to these locations. Although, viral RNA could not be detected at these sites (120), this does not exclude the possibility that undetectable levels of antigen may be present at these sites.  $T_{\text{RM}}$ may also form clusters around local dendritic cells that are still presenting antigen from a previous infection. This hypothesis is supported by the observation that antigen presentation by bone marrow-derived-APCs was able to support CD103 expression by T<sub>RM</sub> (120).

#### **OTHER VIRUSES**

Polyomaviruses are opportunistic pathogens that usually remain latent following infection. However, in immunocompromised individuals, infection can cause multifocal leukoencephalopathy (122). T<sub>RM</sub> are generated in the context of polyomarvirus infection (123-126), and polyomavirus-specific brain CD8+ T<sub>RM</sub> in mice maintain a high TCR affinity for pathogen epitopes. In fact, T<sub>RM</sub> TCR affinity appears to be higher than the TCR affinity of T cells from the spleen. This observation supports a role of  $T_{RM}$  in mediating rapid control of viral replication during reactivation, as high TCR affinity allows for the early detection of low amounts of virus (123). In contradiction to this finding, evidence from another study suggests that lower TCR stimulation increases the generation of brain  $T_{RM}$  (125). One way of interpreting these seemingly contradicting observations is that brain  $T_{\text{RM}}$ initially differentiate from circulating effector T cells with low TCR stimulation capacity, but after taking residence in the brain, undergo functional avidity maturation (127) increasing their ability to respond to antigen. A renal transplant clinical study suggests that renal BK Polyomavirus-specific  $T_{RM}$  were rendered incapable of protecting against infection leading to interstitial nephritis, likening these  $T_{\mbox{\tiny RM}}$  to dysfunctional tumor-infiltrating lymphocytes (126).

Ebola virus causes a form of hemorrhagic fever characterized by intravascular coagulation and maculopapular rash. Although the natural reservoirs for the virus are thought to be fruit bats, human-to-human transmission can occur when contaminated body fluids breach mucosal barriers or skin. Absence of specific treatment and epidemic potential of the virus highlights the need for a vaccine (128). Aerosol administration of a human parainfluenza virus type 3-vectored vaccine expressing an Ebola envelope glycoprotein was capable of not only eliciting neutralizing antibodies but also a CD103<sup>+</sup> T cell response in the lungs of macaques. A large proportion of these T<sub>RM</sub> were polyfunctional, demonstrating positivity for two or more activation markers. Furthermore, a single dose of this vaccine conferred 100% protection against infection challenge (129). Since a large proportion of transmission in the recent Ebola epidemic was through skin contact, vaccination *via* scarification is worth exploring.

Norovirus is a highly infectious virus, and is a common cause of gastroenteritis. Although infection is generally self-limiting, chronic forms have been reported in immunocompromised patients. A clinical study has implicated CD8<sup>+</sup> T cells resembling  $T_{RM}$  in the immune response against norovirus (130). However, a genetically manipulated strain of murine norovirus causing chronic infection revealed that despite a robust and functional  $T_{RM}$  response being generated, clearance of the virus was not achieved, likely due to inadequate antigen sensing (131).

# THE ROLE OF $T_{\mbox{\tiny RM}}$ IN BACTERIAL INFECTIONS

Although there is significantly less literature about  $T_{RM}$  in the context of bacterial infections, the evidence largely implies that  $T_{RM}$  have a noteworthy role in protecting against pathogenic bacteria. The following section groups bacterial pathogens together depending on their location of primary infection.

## BACTERIAL INFECTIONS OF THE LUNGS AND AIRWAYS

Pertussis, also known as whooping cough, is caused by Bordetella pertussis, a Gram-negative coccobacillus. Despite high vaccination coverage, whooping cough remains a serious public health concern. T cell responses are critical for immunity against B. pertussis (132). While the existing whole-cell pertussis (wP) vaccine is generally associated with a strong Th1 response, immunization with the widely used acellular pertussis (aP) vaccine induces a Th2-dominated humoral response (133). Immunity to the aP vaccine wanes over time compared to wP vaccines (134). This diminished immunity allows for the transmission of *B. pertussis* to susceptible individuals. A recent study reported that following B. pertussis infection, IL-17- and IFN- $\gamma\text{-secreting CD4}^+$   $T_{\text{\tiny RM}}$  congregate in the lungs of infected mice where they persisted for 120 days, and expanded up to sixfold upon reinfection. Egress inhibitor FTY720 did not affect the control of bacterial burden during secondary infection, suggesting that  $T_{\mbox{\tiny RM}}$  were capable of providing immunity irrespective of peripheral T cell recruitment. Bacterial clearance in reinfected mice also correlated with CD4+ T<sub>RM</sub> expansion, with a large portion of cells displaying a Th17 phenotype (135).

July 2018 | Volume 9 | Article 1574

 $T_{\mbox{\tiny FM}}$  in Infectious Diseases and Vaccines

Adoptive transfer of lung CD4<sup>+</sup> T<sub>RM</sub> from infected mice into naïve hosts conferred protection against *B. pertussis* challenge (135), suggesting that Th17-like CD4<sup>+</sup> T<sub>RM</sub> seemed to play a crucial role in long-term immunity. Interestingly,  $\gamma\delta$  T cells that express CD69 and CD103, classically known to provide innate-like protection during primary infection, also provided a significant early-release IL-17 response during secondary infection in convalescent mice. However,  $\gamma\delta$  T<sub>RM</sub>, especially V $\gamma4^+$   $\gamma\delta$  T cells persisted in the lungs of convalescent mice and produced a greater IL-17 response on re-exposure to *B. pertussis* in an antigen-specific manner (136). Therefore, a long-lasting *B. pertussis* vaccine should not only promote the generation of *B. pertussis*-specific CD4<sup>+</sup> T<sub>RM</sub> but also  $\gamma\delta$  T<sub>RM</sub>.

Pneumonia is one of the largest infectious causes of mortality in children worldwide (137). The most common cause of community-acquired pneumonia is Streptococcus pneumoniae, a Gram-positive polysaccharide-encapsulated bacterium (138). Modern pneumococcal vaccines are polysaccharide based and are thus poorly immunogenic, providing serotype-specific immunity that wanes over time. Although CD4+ Th17 responses are considered vital in providing protection against pneumococcal infections, the role of  $T_{\text{RM}}$  is yet to be fully characterized. Experimental S. pneumoniae infection was found to promote the production of heterotypic CD4+  $T_{\text{\tiny RM}}$  of both Th17 and Th1 phenotypes in niches located within pneumonia-affected lobes of the lung. It was also observed that immunity was restricted to pathogen-experienced tissue, suggesting that T<sub>RM</sub> reside in primary infection sites, rather than providing immunosurveillance throughout the entire respiratory mucosa. Despite spatial restriction,  $T_{\mbox{\tiny RM}}$  provided superior protection to the local tissue when compared to systemic immune responses elicited by antigen-specific CD4+  $T_{\text{CM}}.$  Neither adoptive transfer of splenic CD4+ T cells from infected mice into naïve recipients, nor inhibiting lung translocation of circulating CD4+ T cells with FTY720 in pathogen-experienced mice, had a significant effect on protection against pneumococcal infection challenge. Therefore, protective immunity against bacterial pneumonia is likely due to the aggregation of CD4+ T<sub>RM</sub> in susceptible tissues (139). Interestingly, combining whole virion influenza and whole cell pneumococcal vaccine also promoted the generation of lung  $CD4^+$  T<sub>RM</sub>. It is likely that these T<sub>RM</sub>, in combination with the accompanying high antibody titers elicited by the combined vaccine, played a role in providing protection against pneumococcal-influenza co-infection (140). Overall CD4 $^+$  T<sub>RM</sub> may play a role in the generation of naturally acquired immunity against pneumococcal infections, and should be considered in the development of heterotypic pneumococcal vaccines.

Mycobacterium tuberculosis (*Mtb*), an acid-fast staining intracellular bacterium, is the causative agent of tuberculosis (TB). The deadly infection can present as pulmonary, as well as extra pulmonary disease (141). Currently, *Bacillus Calmette-Guérin* (BCG) is the only licensed vaccine against TB, and prevents dissemination in children. However, BCG does not provide strong enough immunity against pulmonary TB in adults, therefore, allowing transmission (142). Immune control of *Mtb* infection largely relies on the production of IFNγ by CD4<sup>+</sup> T cells, which enhances macrophage killing of persisting intracellular *Mtb* and leads to the formation of granulomas around sites of bacterial replication (141). A clinical study revealed that individuals previously exposed to tuberculosis were likely to have a population of lung-resident Th1 effector memory cells that released IFN- $\!\gamma$  in response to Mtb antigen re-exposure (143). However, the delay in activation and recruitment of TB-specific T cells to the lungs during primary infection allowed for *Mtb* to proliferate, resulting in a high bacterial burden. The importance of airway-residing memory T cells (then called airway luminal cells) in mediating protection against TB has been described well before the dawn of T<sub>RM</sub> (144-146). However, these cells most likely represent the same cell type. Lung  $T_{\mbox{\tiny RM}}$  induced by mucosal vaccination have shown to be effective in limiting the early control of bacterial replication (147). Despite the defined role of CD4<sup>+</sup> T cells in controlling TB, recent evidence from vaccine studies suggest that CD8<sup>+</sup> lung  $T_{\text{RM}}$  also play an important role in protection against Mtb (148, 149). Only mucosal administration of BCG led to the generation of airway  $T_{\mbox{\tiny RM}}$  that produce higher levels of pro-inflammatory cytokines, including IFN- $\gamma$  than CD8+  $T_{\text{EM}}.$ Furthermore, adoptive transfer of sorted airway CD8<sup>+</sup> T<sub>RM</sub> from BCG-vaccinated mice demonstrated enhanced protection against  $\mathit{Mtb}$  challenge in recipient mice. Transfer of CD8+  $T_{RM}$  decreased the numbers of alveolar macrophages, while increasing the number of CD4<sup>+</sup> T cells and B cells in the infected lung tissues (148). It was hypothesized that CD8+ T<sub>RM</sub> kill Mtb-infected alveolar macrophages, thereby depleting intracellular reservoirs of the bacteria and limiting the entry into the lung parenchyma (148). Likewise, the viral-vectored vaccines SeV85AB and AdAg85A, administered via the intranasal route have also shown to elicit an immune response that favors the production of CD8+ rather than CD4<sup>+</sup> T<sub>RM</sub> (149, 150). In a rhesus monkey model, a cytomegalovirus vector delivering a range of *Mtb* antigens (RhCMV/TB) provided significant protection against tuberculosis, presumably through it its ability to generate and maintain pathogen-specific CD4+ and CD8+ circulating and more importantly resident memory T cells that selectively express VLA-1 (151). Finally, aerosol vaccination with an attenuated Mtb strain lacking sigH not only led to an enormous influx of T cells expressing CD69 into the lung airways (likely to include TRM), but also to a significant long-term protection against virulent Mtb challenge (152). Collectively, these studies indicate that rationally designed TB vaccines should generate immune responses that prevent the establishment of infection and/or provide sterilizing immunity by inducing both lung CD4<sup>+</sup>  $T_{RM}$  and CD8<sup>+</sup>  $T_{RM}$  in the lungs.

# BACTERIAL INFECTIONS OF THE UROGENITAL TRACT

A number of bacteria cause disease of the reproductive tract and urinary system. One such example is Ct, an obligate intracellular bacterium that causes infections of the genitals and eyes. It is the leading cause of infectious blindness worldwide, and can cause infertility when sexually transmitted (153). According to clinical evidence, it appears that spontaneous clearance of clinical infection correlates with at least partial protection against *C. trachomatis* through the production of INF- $\gamma$ -secreting cells

such as CD4<sup>+</sup> Th1 cells. However, IFN- $\gamma$  responses alone do not seem to provide complete protection. It has also been documented that B cell-antibody responses are involved in immunity, especially against secondary infection (154-156). Importantly, intraepithelial CD8+ lymphocytes and MLCs composed of B cells and CD4<sup>+</sup> T cells border the vaginal and uterine tract, respectively in pathogen-experienced tissue. These immunocyte structures hinder C. trachomatis from replicating and establishing a clinical infection (157). Optimal protection against Chlamydia requires both the recruitment of  $T_{\mbox{\tiny CM}}$  and the presence of  $T_{\mbox{\tiny RM}}$  within the urogenital tract (158, 159). It is likely that protective immunity occurs in response to chronic or repeated infection, which leads to the seeding of  $T_{RM}$  throughout the epithelial surface (160). A vaccine composed of Chlamvdia major outer membrane protein and ISOCMATRIX adjuvant was able to provide enough protection to prevent the sexual transmission of C. trachomatis, however, was not capable of providing complete immunity. This may be attributed to the inability of the vaccine to generate a large enough  $T_{\mbox{\tiny RM}}$  population, underscoring the essential role of  $T_{\mbox{\tiny RM}}$  in Chlamydia infection (159). In a separate study, mice were either inoculated with infectious C. trachomatis or UV-inactivated C. trachomatis (UV-Ct). Mice infected with the infectious form demonstrated capacity to control future infections better than naïve controls, which may be attributable to the production of both Chlamydia-specific  $T_{\mbox{\tiny CM}}$  and  $T_{\mbox{\tiny RM}}$  populations. However, the group of mice inoculated with UV-Ct suffered higher bacterial burdens when compared to naïve controls. This data in conjunction with the generation of de novo Ct-specific  $T_{\mbox{\scriptsize reg}}$  suggest that a tolerogenic immune response occurred in these mice. On the contrary, intra-uterine administration of UV-Ct conjugated with charge-switching synthetic adjuvant peptides (UV-Ct-cSAP) conferred a superior protection to Ct in both conventional and humanized mice. The rapid clearance of Ct in UV-Ct-cSAPvaccinated mice has been attributed to the immediate release of IFN- $\gamma$  by mucosal T<sub>RM</sub> (158). Taken together, the ideal vaccine against Chlamydia should promote the generation of local MLC,  $T_{\mbox{\tiny CM}}$  , and  $T_{\mbox{\tiny RM}}$  in the epithelium, even though partial protection appears to be sufficient to prevent disease transmission.

# BACTERIAL INFECTIONS OF THE GASTROINTESTINAL TRACT

Gastrointestinal infections are generally acquired through ingestion of contaminated food or water. These bacteria may remain in the gut, or may disseminate to other parts of the body causing systemic disease (161). Targeted induction of  $T_{\rm RM}$  along the gastrointestinal epithelium could enhance protection against these pathogens. *Listeria monocytogenes*, a food-borne Gram-positive coccobacillus, is of particular concern in immunocompromised and pregnant individuals, and can cause meningitis and stillbirth (162). Its capacity to replicate within host cells facilitates immune evasion. Thus, protection against *L. monocytogenes* is largely dependent on cell-mediated immunity (163). First observed in 1981 as a distinct population of long-lived T memory cells "positioned" in tissue following listeriosis (164), a more recent study highlighted the role of intestinal CD8<sup>+</sup> T<sub>RM</sub> in mediating

immunity against *L. monocytogenes* following oral infection. In fact, blockage of integrin  $\alpha_4\beta_7$  prevented the formation of these intestinal TGF- $\beta$ -dependent  $T_{RM}$  resulting in diminished protection upon re-challenge (165). Revealed by multi-photon dynamic microscopy, a population of V $\gamma$ 4<sup>+</sup>  $\gamma$ 8  $T_{RM}$  was found within the mesenteric lymph nodes in response to *L. monocytogenes* infection, which remained largely stationary under homeostatic conditions. However, upon re-challenge, activation of these cells resulted in organized clusters around bacterial replication foci where they released IL-17 and subsequently, the recruitment of neutrophils to facilitate bacterial elimination. Similarly to  $\gamma$ 8  $T_{RM}$  function seen in *B. pertussis* infection (136), neutralization of IL-17 hindered bacterial clearance, highlighting the importance of early IL-17 release by  $\gamma$ 8  $T_{RM}$  (166).

Yersinia pseudotuberculosis (Yptb), a food-borne pathogen causing of Far East scarlet-like fever, is a Gram-negative bacterium responsible for gastroenteritis, mesenteric lymphadenitis, and can clinically mimic acute appendicitis (167). A Yptb oral infection mouse model showed a robust CD8+ T cell response in the intestines including a population of Yptb-specific CD103+  $\mathrm{CD8^{\scriptscriptstyle +}}\ \mathrm{T_{RM}}$  uniformly distributed throughout the intestine, while CD103<sup>-</sup>  $T_{\text{RM}}$  formed around sites of primary infection where they carried out effector functions (168). Although found in antigenrich areas, their development is independent of local antigen stimulation (168). The development of T<sub>RM</sub> populations in the intestine seems to rely on inflammatory signals from the site of infection rather than antigens (169). Production of IFN-β and IL-12 from intestinal macrophages effectively suppresses TGF-βmediated CD103 expression thereby leading to the development of CD69<sup>+</sup> CD103<sup>-</sup> T<sub>RM</sub> population in mice during *Yptb* infection. This data suggest a central role for inflammatory monocytes in the differentiation and maintenance of different CD8+ T<sub>RM</sub> populations to achieve optimal protection against intestinal infections. Additionally, this study raises the question as to whether CD103 is necessary for residence within the intestinal tissue, or whether it negatively regulates  $T_{\mbox{\tiny RM}}$  capacity to migrate within their residential tissues.

Salmonella spp. is a group of Gram-negative bacilli that is a common cause of gastrointestinal infections responsible for "food poisoning." Transmitted orally, this heterogeneous group of bacteria contains typhoid or enteric-fever causing serovars that can be potentially fatal to humans (170). Current vaccines against Salmonella are poorly immunogenic and risk disease in immunocompromised individuals (171). An effective vaccine that prevents gastrointestinal infection is much needed to prevent outbreaks of salmonellosis. Subcutaneous co-administration of Salmonella SseB and flagellin has shown to provide protection against systemic disease in mice. However, parabiosis studies suggest that this protection can be transferred via the circulation, diminishing the role of  $T_{RM}$  in the observed immunity (172). Nevertheless, it may be beneficial to assess the capacity of this vaccine and others to protect against gastrointestinal infection by oral administration. However, the barriers of oral tolerance and destruction of vaccine components by digestive enzymes and chemicals must be overcome in order to develop oral vaccines.

In summary,  $T_{RM}$  responses in bacterial infections appear to be more diverse compared to viral infections, an observation that

may be attributed to the varying locations of bacterial replication (intracellular versus extracellular), the more complex lifestyles and the presence or more sophisticated immune evasion mechanisms. Future studies should assess the role of  $T_{RM}$  in the natural immune response to other bacterial infections.

# THE ROLE OF T<sub>RM</sub> IN PARASITIC (PROTOZOA AND HELMINTHS) INFECTIONS

Protozoa are unicellular organisms that are of great importance to human health. Most prevalent in tropical regions of the world, protozoan infections are difficult to treat due to their complex life cycles and their ability to evade host immune responses through antigenic variation, residence within various intracellular compartments, and their capacity to assume protective forms such as cysts (173, 174). At this juncture, our collective understanding of  $T_{RM}$  responses to protozoan infections remains relatively deficient. However, studies have shown that  $T_{RM}$  play a significant role in protecting against a few protozoan species.

Malaria, the most prevalent protozoan infection of humans, is caused by five species of Plasmodium. Transmitted by the bite of an infected female Anopheles mosquito, Plasmodium parasites enter the circulation and take residence inside erythrocytes during part of their complex life cycle. Natural immunity to Plasmodium infection involves a mixture of humoral, CD4+, and CD8<sup>+</sup> T cell responses (175). However, liver T<sub>RM</sub> have emerged as a promising target for protecting against malaria (176). Unlike T<sub>RM</sub> of the epithelium, such as the lungs, intestines, and skin, liver- $T_{\mbox{\tiny RM}}$  appear to reside in sinusoids (the blood vessels of the liver), rather than the parenchymal tissue (177-179). The heavily fenestrated architecture of these blood vessels and the distinct slow flow rate of blood allows for  $T_{\mbox{\tiny RM}}$ to traverse through the organ without being dispatched into circulation. Furthermore, liver sinusoids provide a prime niche for close interaction of T<sub>RM</sub> and antigen presenting cells, such as Kupffer cells and dendritic cells. This allows for the rapid detection of antigen. Each hepatocyte is also in close association with a sinusoid, thereby providing easy access to liver  $T_{\mbox{\tiny RM}}$ for assessment of surface antigen presentation (177). Intravital imaging revealed that these  $T_{RM}$  traversed around 10 µm per minute and, as reported in HSV-1 infection of skin, T<sub>RM</sub> assume an amoeboid form, extending dendrites to survey the liver for antigens (2). Rather than relying on CD103- $\alpha$ E integrin interactions for maintaining tissue residence, liver T<sub>RM</sub> appear to utilize the adhesion molecule LFA-1 (3). A rhesus monkey P. knowlesi infection model that assessed sporozoite immunization demonstrated capacity for generating liver T<sub>RM</sub>. These T<sub>RM</sub> appear to be protective as their depletion resulted in the loss of immunity (1). Experiments with radiation-attenuated sporozoites also support the notion that inducing high numbers of liver T<sub>RM</sub> can afford protection against malaria. In this study, a "prime-and-trap" strategy was used in which primed T cells from the spleen were drawn to the liver using a recombinant adeno-associated virus that infected hepatocytes and subsequently caused them to express *Plasmodium* antigen. The immunity generated by this strategy was attributed to the increased numbers of CD8<sup>+</sup> liver  $T_{RM}$  (2). The emergence of liver  $T_{RM}$  as a potential target for pre-erythrocytic malaria vaccines warrants further research.

Leishmaniasis is a heterogeneous vector-borne disease that is caused by an intracellular protozoa parasite. There are over 20 known leishmania species, all of which are transmitted by bites from infected female Phlebotomine sandflies. Clinical disease presents in three main forms: cutaneous, mucocutanous, and visceral. Subjugation of Leishmania parasites relies on the establishment of IFN- $\gamma$ -producing CD4<sup>+</sup> Th1 cells (180). While it is widely known that clearance of primary infection can lead to protective immunity, the effector response that leads to protection remains unclear. It has, however, become apparent that T<sub>RM</sub> play a crucial role in providing such protection (181-183). L. major infection models illustrated that following infection, long-lived T<sub>RM</sub> are rapidly fabricated and seeded universally throughout the skin, as they can be detected in tissue far away from the primary site of infection (181). It was previously believed that these T<sub>RM</sub> provided immunity by rapidly recruiting circulating effector T cells. However, more recent studies suggest that the recruitment of circulating T cells may not be as important as previously thought, as FTY720 and aCXCR3-treated mice re-challenged with L. major showed minimal difference in early parasite control (182, 183). Furthermore, parabiosis studies demonstrated that Leishmania-specific circulating T cells alone provide little or no protection during early infection. Data exemplifies that CD4<sup>+</sup> T<sub>RM</sub> are rather likely to provide immunity by eliciting a delayed-type hypersensitivity response. Early immunity is attributable to the capacity of  $T_{\mbox{\tiny RM}}$  to rapidly recruit reactive oxygen species/nitric oxide producing inflammatory monocytes to control parasite burden (182). Liver  $T_{RM}$  have also been implicated in the immune response against Leishmania. The recombinant proteins LirCyp1 and LirSOD of L. infantum appear to be good candidates for promoting the expansion of liver memory T cells (184). No current vaccine exists for this potentially fatal disease. Together, these data suggest that Leishmaniasis vaccines should be tailored to generate T<sub>RM</sub> to provide heterotypic protection against the many species that cause disease.

Toxoplasma gondii, the causative agent of toxoplasmosis, is an intracellular protozoan that is generally acquired through contaminated food. In humans, *T. gondii* can form persistent cysts in multiple tissues (185). In both acute and chronic infection, cell-mediated immunity and effector cytokines play vital roles in limiting the progression of disease (186). Mice deficient in TNF-α suffer from increased pathology, and IFN-γ production in the brain stimulates microglia and astrocytes to inhibit protozoan proliferation. In a chronic infection model of *T. gondii*, CD103<sup>+</sup> CD8<sup>+</sup> T<sub>RM</sub> established in the brain produced a more robust IFN-γ and TNF-α response when compared to CD103<sup>-</sup> T cell subsets (187). It appears that brain T<sub>RM</sub> provide superior protection against *T. gondii* infection of the central nervous system when compared to CD8<sup>+</sup> T<sub>EM</sub> and T<sub>CM</sub>. During *T. gondii* and *Y. pseudotuberculosis* infection T<sub>RM</sub> also seem to

accumulate in white adipose tissue in what appears to be a depot of protective memory cells (188).

Helminthic infections are highly prevalent around the world. Although most infections are not fatal, they account for a large proportion of disease burden, causing secondary conditions, such as anemia and malnutrition. Immunity against helminthic infection is largely mediated by the Th2 effector arm of the adaptive immune system (189). The role of T<sub>RM</sub> in protecting against helminthic infections, however, has only been explored recently in two species: Heligmosomoides polygyrus and Nippostrongylus brasiliensis (190, 191). While neither of these species are human pathogens, they provide analogous models to gastrointestinal helminthic infections and Necator americanus infection in humans, respectively (192, 193). Adoptive transfer of peritoneal-cavity CD4+ T<sub>RM</sub> from convalescent mice into naïve mice prior to H. polygrus infection challenge, has demonstrated that peritoneal-cavity derived CD4+ T<sub>RM</sub> are capable of hindering the reproductive capacity of female worms without reducing worm burden (190). This phenomenon provides new insight into what appears to be a unique interaction between T<sub>RM</sub> and pathogen. A different study that used a N. brasiliensis model demonstrated that even a small number of lung-interstitial  $\mathrm{T}_{\scriptscriptstyle RM}$ were capable of providing protective immunity. This was confirmed as cognate mice treated with FTY720 and lymphotoxin beta-receptor fusion protein (which causes lymphopenia) were able to clear secondary infection, suggesting that circulating T cells are not necessary to mount a protective secondary response (191). In spite of the lack of knowledge surrounding the interaction between T<sub>RM</sub> and helminths, there is a clear role for this subset of T cells in worm infections that needs to be explored further.

# THE ROLE OF T<sub>RM</sub> IN FUNGAL INFECTIONS

Typically, fungal infections are less frequent compared to viral and bacterial diseases. However, due to the increasing use of immunomodulatory drugs for cancer and organ transplant patients, the increasing incidence of mycosis is of clinical importance (194). T<sub>RM</sub> responses are least studied in the context of fungal infections. In fact, only one fungus appears to have been used in T<sub>RM</sub> studies.

Candida albicans is a dimorphic yeast and opportunistic pathogen. Although it forms part of the normal commensal biome of humans, it can cause infections known as candidiasis especially in immunocompromised individuals (195). Skin and tongue-resident CD4<sup>+</sup> IL-17-producing T<sub>RM</sub> can provide effective protection against *C. albicans* (196, 197). Murine skin and oral infection models demonstrated that during early infection,  $\gamma\delta$  T cells release IL-17 in response to *C. albicans* (196, 197). In skin, by day 7 post-infection, the vast majority of IL-17-producing cells are of the Th17 phenotype. Eventually, the T cells at the site of initial infection upregulate CD103 and CD69 suggesting they assume the CD4<sup>+</sup> T<sub>RM</sub> phenotype between 30 and 90 days post infection. During this time, the cells first become less motile, eventually "sessile" and localize to the papillary

dermis. However, upon reinfection, these  $T_{RM}$  were capable of rapidly clearing infection and appear to be superior at doing so than circulating  $T_{EM}$ . It was also reported that *C. albicans*specific Th17 cells were found in high numbers in normal human skin (196), and that low doses of *C. albicans* antigen exposure stimulates the production of regulatory skin  $T_{RM}$  that substantially suppress the activity of skin  $T_{EM}$  (198). This may be attributed to the widespread presence of *C. albicans* in human tissue. Resident memory Treg cells may play a protective role in preventing a hyper-inflammatory response to benign *C. albicans* antigen exposure. Although the development of a vaccine against *C. albicans* is not of vital importance, these studies provide an initial insight into understanding  $T_{RM}$  responses to fungal infections. Other fungal infections that would be of interest include tinea, cryptococcosis, and aspergillosis.

#### DISCUSSION

The discovery of T<sub>RM</sub> has enhanced the possibility to develop new and improved vaccines. From the available literature, it appears that the most important factor for generating  $T_{RM}$  is to match the route of vaccination to the route of pathogen entry. In general, these are the mucosal and epithelial barriers that provide the first line of defense against pathogens: the respiratory, gastrointestinal, urogenital mucosa, and integumentary epithelium (Figure 3). Thus, the long-standing method of administering vaccines parenterally may be less effective at conferring optimal protection when compared to the novel mucosal and epithelial vaccine strategies highlighted throughout this review. The "prime and pull" method in which a parenteral vaccine is administered (prime) and an inflammatory agent is applied at a later time point to the desired peripheral tissue (pull) has also proven itself to be an effective vaccine strategy for generating T<sub>RM</sub>. The combination of mucosal vaccination and "pull" strategies may be an avenue worth exploring in future experiments. Another promising vaccine strategy is the use of viral-vectored vaccines. Regardless, it is also imperative to keep in mind the balance that these tissues must constantly strike in inducing a tolerogenic versus effector immune response to antigens, given their dual function in both their physiological roles (respiration, digestion, reproduction, etc.) and in serving as barriers against infection. Additionally, it will be important to consider the local cytokine milieu that influences  $T_{RM}$  generation (199). Generating memory T cells in peripheral tissues with cytolytic effector functions and the capacity to recruit inflammatory cells may pose the risk of inducing a hyper-inflammatory state leading to immunopathology. However, the natural persistence of T<sub>RM</sub> in most peripheral tissues for extended periods of time under homeostatic conditions implies that mechanisms are in place to regulate T<sub>RM</sub> responses. Developing a greater understanding of these mechanisms is vital in creating safe  $T_{\mbox{\scriptsize RM}}\mbox{-}based$ vaccines.

A very appealing aspect of  $T_{RM}$ -based vaccines is that it may be possible to generate heterotypic protection, as shown by studies using influenza infection models. This is vital for protecting against a range of rapidly mutating pathogens, such as HIV, as well as infectious diseases such as leishmaniasis that are caused

#### Muruganandah et al.

T<sub>RM</sub> in Infectious Diseases and Vaccines



by heterogeneous pathogens. Furthermore, the promptitude with which  $T_{RM}$  mediate immune responses is also of great interest when generating protection against infections such as tuberculosis that are capable of establishing latent infections. In order to achieve immediate  $T_{RM}$  protection, it appears that there must be a minimal density of  $T_{RM}$  within peripheral tissues to ensure pathogens are identified and eliminated in a timely manner. This may pose a challenge in developing  $T_{RM}$ -based vaccines as sufficient numbers of  $T_{RM}$  need to be generated and maintained evenly throughout infection-susceptible tissues. The spatial capacity in non-lymphoid tissue to accommodate for  $T_{RM}$  may be limited, and thus establishing the capacity of different tissues has as well as determining the minimum threshold of  $T_{RM}$  needed to provide protection is much needed.

#### CONCLUSION

In summary, the findings of this review largely accentuate the importance of  $T_{RM}$  in protecting against a range of pathogens. Their localization to sites prone to infection appears to give  $T_{RM}$  an enhanced capacity to mount swifter immune responses when compared to circulating memory T cells. Previous vaccine development has been largely centered on the generation of systemic memory response, which at times has shown to be ineffective. The capacity to form tissue-specific immunity through  $T_{RM}$  may shape vaccines of the future. Continuing to foster the growing pool of knowledge about  $T_{RM}$  will help to guide the field of  $T_{RM}$  vaccinology and may lead to the generation of new and more effective vaccines which may help to reduce the incidence of many infectious diseases.

mediate immunity against Bordetella pertussis.

#### **AUTHOR CONTRIBUTIONS**

VM performed the literature search and conducted extraction of data from relevant studies. AK, HS, and SN critically reviewed the literature search. VM and AK wrote the manuscript. All coauthors read and approved the final version of the manuscript.

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18

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19

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21