MOLECULAR ANALYSIS OF INNATE IMMUNITY IN TYPE 1 DIABETES

THESIS SUBMITTED BY

ROBY JAMES JOSE

for the degree of Doctor of Philosophy

in the School of Pharmacy and Molecular Sciences

James Cook University

January, 2014

TABLE OF CONTENTS

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 4

TRAIL MEDIATED PROTECTION OF DIABETES IN NOD MICE

CHAPTER 5

POLYMORPHISM IN A FAS LIGAND SIMPLE SEQUENCE REPEAT AFFECTS TYPE 1 DIABETES IN NOD MICE

CHAPTER 6

GENE ENVIRONMENT INTERACTIONS IN NOD AUTOIMMUNITY

CHAPTER 6.1

Environmental agent mediated modulation of NOD diabetes

STATEMENT

The research contained within this study was performed in the Medical Genomics Laboratory in the Comparative Genomics Centre at James Cook University, Townsville, under the supervision of Professor Alan Baxter. All research procedures reported in this thesis received the approval of James Cook University's animal ethics committee. The data presented is my own work, with all contributions from others clearly stated in the acknowledgements, methods and the body of the thesis.

_____

ROBY JAMES JOSE

ABSTRACT

Introduction: Type I diabetes (T1D) is an autoimmune disease of children and young adults that selectively destroys the insulin-producing islet cells in the pancreas. NOD mice, the best validated animal model of T1D, manifests islet infiltration as early as 4 weeks, leading to β-cell destruction and eventually overt diabetes. This study was designed to determine the role of TRAIL, FASL and IFN-γ, components of innate immune system, in the biology of β-cell destruction in NOD mice.

Experiments: To test the hypothesis that differential expression of TNF superfamily ligands (TRAIL and FASL) affects tolerance mechanisms, I assessed the diabetes susceptibility of TRAIL-deficient NOD.*Tnfsf10*$^{-/-}$ mice and of mice which exhibited allelic *Fasl* expression (NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2}). Immune cells from these three mouse lines were systematically characterised by flowcytometry, and their diabetogenic potential determined by adoptive transfer experiments. β-cell response in a TRAIL deficient environment was assayed in islet cultures. Micro array analysis was performed to compare the β-cell response in mice with allelic *Fasl* expression. To investigate the role of IFN-γ in NOD diabetes, I assessed the diabetes susceptibility of targeted gene deficient mutants of *Ifng* (NOD.*Ifng*$^{-/-}$) and the β chain of *Ifng* receptor (NOD.*Ifngr2*$^{-/-}$). I examined the role of IFN-γ in BCG-induced diabetes protection by assessing diabetes susceptibility of

BCG treated NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice, and by characterising their immune cell compartments. Further, the contribution of IFN-γ in accelerated precipitation of lupus in NOD mice was studied by administering 1mg BCG to NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice.

Results: Targeted deletion of TRAIL resulted in increased severity of spontaneous insulitis and an increased incidence of diabetes. This was the first ever demonstration of an increased incidence of spontaneous diabetes in NOD mice bearing a targeted deletion of TRAIL. Similarly, florid lymphoid infiltrates and high diabetes incidence in NOD.*D1Bax208*^{m2} mice, which exhibited low FASL-expression, provided direct evidence for a protective role of this death ligand in autoimmune diabetes. Despite increased susceptibility to autoimmunity, tolerance mechanisms in these mouse strains were unaffected. In adoptive transfer experiments, numbers of diabetogenic T cells were found to be unchanged in NOD.*Tnfsf10*$^{-/-}$ and NOD.*D1Bax208*^{m2} donor mice, whereas NOD.*Tnfsf10*$^{-/-}$ and NOD.*D1Bax208*^{m2} recipient mice showed increased susceptibility to diabetes. These findings raised the possibility of TRAIL and FASL expression in the nonhematopoietic compartment conferring protection against T1D. LPS activation of NOD.*Tnfsf10*$^{-/-}$-islets and comparison of gene expression profiles between NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice indicated that TRAIL and FASL regulated the islet-expression of the potent chemokine, Interferon-γ-induced protein-10 (IP-10) and the suppressor of cytokine signalling (SOCS1), a strong inhibitor of IFN-γ signalling. TRAIL and FASL-mediated suppression of IP-10, and upregulation of *Socs1* transcription, rendered the NOD-islets less susceptible to

immune destruction in T1D. Proinflammatory cytokine IFN-γ was not essential for diabetes development in NOD mice, but was vital for BCG-mediated diabetes protection. Diabetes incidence in NOD.*Ifng*$^{-/-}$ mice was similar to that of WT NOD/Lt mice, however, BCG failed to inhibit the disease in mice lacking IFN-γ-signalling. IFN-γ-induced elimination of effector T cells played a role in BCG-mediated diabetes protection in NOD mice. At a high-dose of 1mg, BCG precipitated a systemic form of autoimmunity that had characteristics similar to human SLE, in these mice. However, I found that deficiency in IFN-γ-signalling abrogated the influence of BCG in mediating the expression of NODs tendency towards autoimmunity.

Conclusion: This study assessed the mechanism of protection mediated by TRAIL, FASL and IFN-γ, components of the innate immune system, in NOD diabetes. I observed a remarkable parallel between the β-cell protective effects of TRAIL and FASL, the two homologous TNF superfamily ligands. An exciting role for the proinflammatory cytokine IFN-γ, in modulating NOD diabetes in a BCG-mediated T1D-protection model, was also identified. Results discussed here provide insight into the islet immune specific regulatory role of TRAIL, FASL and IFN-γ in the pathogenesis of diabetes and suggest therapeutically beneficial strategies for islet preservation.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Professor Alan Baxter for his time, expert opinion and for providing me with an excellent atmosphere to undertake this project. I would like to thank Professor Heinrich Korner and Dr. Michael Ridd for their invaluable inputs and inspiring chats. Special thanks to Professor Jonathan Golledge for offering great support during my thesis-writing phase.

Thank you to all my colleagues at the medical genomics group for their support and encouragement, without which, most of this work would not have been possible. I greatly benefitted from Dr. Margaret Jordan's expertise in molecular genetics and Ms Julie Fletcher's knowledge of flowcytometry. Islet isolation was performed with much appreciated help from Dr. Shahead Chowdhury (JCU) and Ms Stacey Walters (Garvan Institute of Medical Research, Sydney). I also want to thank Dr. Erik Biros, Dr. Nicole Gerlach, Ms Socorro Miranda-Hernandez and Ms Alicia Jade for helping-out in large FACS experiments. Special thanks to Dr. Florian Weide and Dr. Phillip Fromm for being there, even at odd times, to give tips and help fix the endless problems with the wonderful but temperamental flowcytometer. Deep thanks to the wonderful staff of the Immunogenetics Research Facility for taking great care of all the mouse lines, on which my work was based. I am sincerely grateful to our collaborators, Professor Mark Smyth (Peter MacCallum cancer centre, Melbourne) and Professor Thomas Kay

(St. Vincent's institute of medical research, Melbourne), for kindly providing the TRAIL-deficient mice. A warm thank you to Dr. Venkat Vangaveti of the Obesity Group and Dr. Smriti Krishna of the Vascular Biology Unit, both at the School of Medicine, for their companionship, encouragement and support.

Further acknowledgements are owed to the Commonwealth for awarding me the International Postgraduate Research Scholarship and School of Pharmacy and Molecular Science for the top-up scholarship and graduate research scheme awards. I thank the Logan Foundation for awarding me a travel grant to attend the ASI annual conference in Canberra. Thanks to Federation of Immunological Societies of Asia (FIMSA) and Juvenile Diabetic Research Fund (JDRF) for funding me to attend international conferences.

Last but not the least; I would like to thank my lovely wife Nidhi and our cutie pies Rhea and Deeya for putting up with me!

LIST OF TABLES

LIST OF FIGURES

……

CHAPTER 1

……

INTRODUCTION

It is estimated that over 20 million people aged between 14 and 31 live with type 1 diabetes (T1D), a lifelong autoimmune disease caused by the progressive loss in the ability to produce insulin. There is no cure for T1D, with patients requiring life-long insulin treatment. The complications of this disease are severe, and include kidney failure, nerve damage, blindness, heart disease and stroke. However, advancements in medical research shows promise in developing therapies and technologies that could improve the day-to-day management and quality of life of T1D patients. Most current therapeutic strategies for diabetes management focus on ameliorating the effects of autoaggressive T cells that infiltrates the islets, including anti-CD3 therapy. It is believed that the disease process is initiated much earlier in life and probably triggered by an environmental agent in a genetically predisposed individual. With increasing evidence suggesting an important role for innate immune mechanisms in the pathogenesis of T1D, components of this arm of the immune system might prove to be beneficial therapeutic targets.

Sophisticated mouse models are available, that could help uncover the molecular pathways of innate immunity linked to T1D development. First section of this study aims at understanding the role of key members of the TNF superfamily members, TRAIL and FASL in NOD diabetes development. In the second section, the role for proinflammatory cytokine IFN-γ in NOD diabetes will be investigated. This study will also illustrate how an environmental agent modulates the immune system to inhibit diabetes in an IFN-γ-dependent manner. Findings from this

research should identify therapeutic targets that are capable of suppressing β-cell destruction.

Study 1	**Aim:** To investigate the role of TNF superfamily ligands TRAIL (*Tnfsf10*) and FASL (*Tnfsf6*) in NOD diabetes inhibition. **Design:** Incidence of spontaneous diabetes and severity of pancreatic islet-infiltration will be assessed in targeted gene deficient mutants of *Tnfsf10* (NOD.*Tnfsf10*$^{-/-}$) and in NOD mice carrying the mutant alleles of *D1Bax208* (NOD.*D1Bax208*^{m1} & NOD.*D1Bax208*^{m2} mice, which exhibits allelic *Fasl* expression). Immune cells of these mice will be characterised by flowcytometry and their diabetogenic potential determined by adoptive transfer experiments. β-cell responses in a TRAIL-deficient environment will be assayed *in vitro*. Islet-expressed genes will be profiled to determine the β-cell response in mice with allelic FASL expression. **Goal:** To identify potential candidates of clinical relevance by determining mechanisms of TRAIL/FASL-mediated protection against islet destruction.
Study 2	**Aim:** To examine the role of IFN-γ in the initiation and development of NOD diabetes and to explore the mechanisms by which *M.bovis* (BCG) inhibit it. **Design:** Incidence of spontaneous diabetes will be assessed in targeted gene deficient mutants of *Ifng (*NOD.*Ifng*$^{-/-}$) and the β chain of *Ifng* receptor (NOD.*Ifngr2*$^{-/-}$). Effects of BCG instillation in NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice will be assayed by observing their diabetes incidence and characterising their immune cell compartments. **Goal:** To unravel IFN-γ-associated mechanisms, which could potentially abrogate insulitis and promote reversion from clinically overt diabetes.

Table 1.1 Overall aims, study design and goals of this thesis

.....

CHAPTER 2

.....

LITERATURE REVIEW

2.1 Innate immune system and autoimmunity

The innate immune system is a universal, evolutionarily ancient and immediate but nonspecific form of host defence against infections. The components of the innate immune system, including pattern-recognition receptors (PRRs) macrophages, dendritic cells (DC), natural killer (NK) cells, Natural Killer T (NKT) cells and γδ-T cells and soluble factors like complement, cytokines, chemokines, acute phase proteins etc. functions as the first line of defence against invading microbes. They also contribute to the activation of a highly specific but temporally delayed adaptive immune response. Innate and adaptive arms of the mammalian immune system continually strive towards the efficient recognition and elimination of a broad range of invading microorganisms, while causing minimal damage to self. However, in some instances, due to an unfortunate combination of events, the immune system plunges into disarray and begins to operate in a self-destructive manner. Autoimmunity is the failure of an organism to recognize its own constituents as self, thereby allowing an immune response against own cells and tissues. Any disease that arises from such aberrant immune response is termed autoimmune disease. Autoimmune disorders encompass a wide spectrum of diseases, which are grouped clinically based on their activity, into organ-specific and systemic disorders (Table 2.1). Some of these diseases are characterised by autoantibody production while others are distinguished by anomaly in the selection, regulation or deletion of T/B cells (Table 2.2).

A. Organ specific autoimmune disorders

Disease	Target Organ	Antibody against
Hashimoto's Thyroiditis	Thyroid cells	Thyroglobulin/ thyroid peroxidase
Graves' disease	Thyroid follicular cells	TSH receptor
Addison' disease	Adrenal cortex	Adrenal cells having 21-hydroxylase
Spontaneous male infertility	Sperms	Sperm antigen
Type 1 Diabetes	Pancreas	Pancreatic islet β-cells
Myasthenia gravis	Muscle	Muscle, acetylcholine receptor
Goodpasture's Syndrome	Kidney, Lung	Renal & lung basement membrane

B. Systemic autoimmune disorders

Disease	Target	Antibody against
Pemphigoid	Skin basement membrane	protein type XVII collagen/BP180
Idiopathic thrombocytopenia	Platelet	Platelet
Primary biliary cirrhosis	Liver	Mitochondria
Idiopathic neutropenia	Neutrophils	Neutrophils
Ulcerative colitis	Colon	Colon lipopolysaccharide
Sjogren's syndrome	Secretory glands	Duct tissue
Vitiligo	Skin	Melanocytes
Rheumatoid arthritis	Joints	IgG
Systemic Lupus Erythematosus (SLE)	Skin, Kidney, Joints red blood cells etc.	DNA, RNA, nucleoproteins, RBC
Pernicious anaemia	Red cells	Intrinsic factor, Gastric parietal cell

Table 2.1 Some of the major organ specific (A) and systemic (B) autoimmune disorders with their target organs and autoantibodies produced.

A. Antibody mediated autoimmune disorders

Disease	Autoantigen	Symptoms
Addison' disease	Adrenal cells having 21-hydroxylase	Haemolysis, low BP, hypoglycaemia
Autoimmune haemolytic anaemia	Rh blood group antigens, I antigen	Lysis of RBC by complement and FcR^+ cells, anaemia
ITP	Platelet integrin GpIIb:IIIa	Abnormal bleeding
Goodpasture's syndrome	basement membrane Type IV collagen	Glomerulonephritis, pulmonary haemorrhage
Graves' disease	Thyroid-stimulating hormone receptor	hyperthyroidism
Hashimoto's thyroiditis	Thyroglobulin, thyroid peroxidase	hypothyroidism
Myasthenia gravis	a chain of nicotinic acetylcholine receptor	Progressive muscle weakness
Rheumatoid arthritis	Rheumatoid factor IgG complexes	Arthritis
SLE	DNA, histones, ribosomes, snRNP, scRNP	Glomerulonephritis, vasculitis

B. Cell mediated autoimmune disorders

Disease	Autoantigen	Symptoms
EAE, MS	Myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein	Brain invasion by CD4 T cells, weakness
Hashimoto's thyroiditis	Thyroid antigen(s)	Thyroid under-activity
Type 1 diabetes	Pancreatic β-cell antigen(s)	β–cell destruction
Rheumatoid arthritis	Unknown synovial joint antigen	Joint inflammation, destruction

Table 2.2 Classification of autoimmune disorders based on the mechanism of tissue damage (Antibody-mediated (A), cell-mediated (B)).

Type-1 diabetes (T1D), multiple sclerosis (MS), rheumatoid arthritis (RA), Crohn's disease and systemic lupus erythematosus (SLE/lupus) are among the more than 100 identified autoimmune diseases. These disorders affect around 5-10% of the developed world's population and are a significant cause of chronic illness and death (Cooper et al., 2009a; Shapira et al., 2010; Shoenfeld et al., 2008a). Single or multiple genetic risk factors and/or environmental factors such as infections, tobacco use, UV exposure, nutrition, xenobiotics etc. are involved in the breakage of self-tolerance, leading to autoimmune disease development (Baxter et al., 1994; Kivity et al., 2009; Mackay, 2009; Pender, 2003; Rieger and Gershwin, 2007; Shoenfeld et al., 2008b).

A complex link exists between environmental agents and the penetrance of autoimmune disease, with the infectious events more likely to affect disease penetrance in genetically predisposed individuals. The role of microbial agents in the pathogenesis of autoimmunity has been extensively studied; while some of these agents trigger various forms of autoimmunity, others are known to suppress the disease (Tables 2.3 and 2.4). Evidence suggest that perinatal infections are associated with an increased risk of developing T1D, while multiple infections during the first few years of life decreases the risk, indicating a sophisticated relationship between environmental factors and the penetrance and expression of genetically determined autoimmune disorders (EURODIAB, 2000; McKinney et al., 1999; Wasmuth et al., 2000).

Organism	Disease Association	Mechanism	Reference
Coxsackie B4 virus	T1D	Bystander damage	(Horwitz et al., 1998)
Rotavirus	T1D	Molecular mimicry	(Honeyman et al., 2000)
Kilham rat virus	T1D	TLR 9 signalling	(Zipris et al., 2007)
Hepatitis B virus	SLE	Molecular mimicry	(Tudela et al., 1992)
Parvovirus	T1D	Change Treg balance	(Tirabassi et al., 2010)
Parvovirus B-19	SLE	Molecular mimicry	(Severin et al., 2003)
EBV	MS or SLE	Autoreactive B cells	(Pender, 2003)
Hepatitic C virus	Myasthenia gravis	Cross-reactivity	(Eddy et al., 1999)
	Autoimmune hepatitis	HCV mediated alteration of hepatocellular LKM1 target antigen	(Lenzi et al., 1990)
	Cryoglobulinemic vasculitis	B cell expansion, autoantibody & IC	(Ferri et al., 2003)
	Rheumatoid diseases	Immune response to HCV	(McMurray and Elbourne, 1997)
Herpes virus 6	MS	Molecular mimicry	(Cermelli et al., 2003; Tejada-Simon et al., 2003)
Coxsackie Virus strains B3 & B4	Autoimmune myocarditis, Sjogren's syndrome	B and T lymphocytic infiltrates	(Baboonian et al., 1997; Triantafyllopoulou et al., 2004)
HSV type 1	Autoimmune Keratitis	Molecular mimicry	(Zhao et al., 1998)
Rubella virus	T1D	Congenital infection	(Ou et al., 2000)
Cytomegalovirus	T1D	Molecular mimicry	(Tirabassi et al., 2010)
Helicobacter pylori	autoimmune gastritis	Cross reactive gastric T cells	(Amedei et al., 2003; Appelmelk et al., 1998)
Streptococcus pyogenes	Acute rheumatic fever Sydenham's chorea	Molecular mimicry	(Kirvan et al., 2003)
Novospingobium aromaticivorans	Biliary cirrhosis	Molecular mimicry	(Selmi and Gershwin, 2004)
Borrelia burgdorferi	chronic autoimmune diseases	immune response to spirochete	(Singh and Girschick, 2004)
Bacillus Calmette-Guérin (BCG)	SLE	Adjuvant effect; Reactivity to 65kDa heat shock protein	(Baxter et al., 1994; Karopoulos et al., 1995; Rodrigues et al., 1990)

Table 2.3 Environmental agents that trigger or amplify autoimmune disease

Organism	Disease Association	Mechanism	Reference
Lymphocytic choriomeningitis virus (LCMV)	T1D (NOD and RIP-LCMV mouse models)	Extensive IP-10 expression in pancreatic draining lymph nodes	(Christen et al., 2004; Oldstone, 1988)
B Coxsackievirus (CVB3)	T1D (NOD)	Immune response against CVB3	(Tracy et al., 2002)
Schistosoma mansoni	T1D (NOD)	Modulation of DC & increase in NKT cell numbers	(Zaccone et al., 2003)
BCG	T1D (NOD)	Generation of suppressor cell types	(Harada et al., 1990)
Murine Hepatitis Virus (MHV)	T1D (NOD)	Immune response against MHV	(Wilberz et al., 1991)
BCG	EAE (C57BL/6)	Shared T cell epitopes; Redirected trafficking of activated T cells	(Ben-Nun et al., 1995; Sewell et al., 2003)

Table 2.4 Environmental agents that ameliorate or inhibit autoimmune disease

2.2 Type 1 Diabetes

Type 1 Diabetes (T1D) is an organ specific autoimmune disorder resulting from the progressive and specific destruction of insulin producing pancreatic β-cells by auto-reactive T cells, culminating in loss of insulin secretion leading to hyperglycaemia and secondary complications including neuropathy, nephropathy, blindness, and cardiovascular disease. Recent studies in animal models and in humans suggest that both innate and adaptive immune responses participate in T1D pathogenesis (Wen et al., 2008; Zipris, 2008).

2.2.1 Epidemiology

Accounting for about 10-15% of all types of diabetes, T1D currently affects over 35 million people (in all age groups) worldwide with its incidence increasing at the rate of 3% every year (Danaei et al., 2011; IDF, 2011; Onkamo et al., 1999). According to the International Diabetes Federation estimate over 490,000 children worldwide had T1D with over 78,000 new cases being reported each year (IDF, 2011 http://www.idf.org/diabetesatlas). T1D mostly arises in the first 30 years of life (usually in childhood) and its complications such as cardiovascular disease, kidney failure and vascular and neuropathic disease significantly shorten life span (Chase et al., 2004; Rewers, 1991). Those with a disease onset under the age of fifteen have a death rate over two times higher than that of general population (Patterson et al., 2007; Skrivarhaug et al., 2006). The World Health Organization (WHO) Diamond project group in a recent study found that global incidence of childhood diabetes incidence consistently escalated during the latter part of the 20th century, compared to the relatively low increment rate at the beginning of the century (DIAMOND, 2006). In the same study, they reported a 350-fold variation in the rate of diabetes development across the 100 populations worldwide, which reflects the global distribution of major ethnic populations. T1D rates are highest among northern European Caucasian populations, and in North America, Australia, and New Zealand, where European Caucasians settled in (Figure 2.1). Analysis of 15-year incidence data from 17 European countries predicted the doubling of new cases of T1D in young children (< 5 years), with a 70% increment in the

prevalence of the disease in those younger than 15 years (Patterson et al., 2009). Data available from South Asian countries, home to more than 20% of world's population indicate a steady increase of diabetes incidence, suggesting an increased global presence of this disease in future.

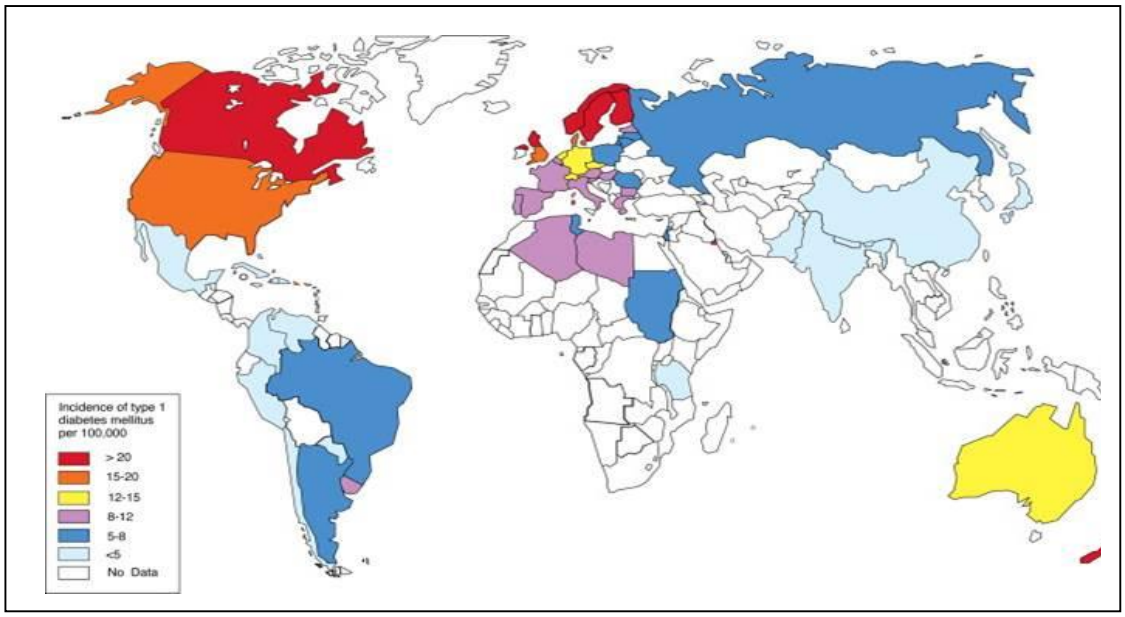


Figure 2.1 Geographical distribution of T1D (adapted from Shapira et al., 2010)

2.2.2 Etiology

The etiologic agents that precipitate the autoimmune process and β-cell destruction are not well established. Recent data suggests that autoimmune disease arise from an unfortunate combination of environmental factors, genetic susceptibility and stochastic events in the immune system (Figure 2.2). Environmental agents including, microbial infection (Tables 2.3 and 2.4), UV-exposure, dietary factors, and xenobiotics are implicated in the pathogenesis of

autoimmune diabetes (EURODIAB, 2000; Goldberg and Krause, 2009; Littorin et al., 2006; Shoenfeld et al., 2008b; Sloka et al., 2010). Large population based studies aimed at establishing an association between environmental factors and T1D have not yielded consistently positive results, underscoring its complexity.

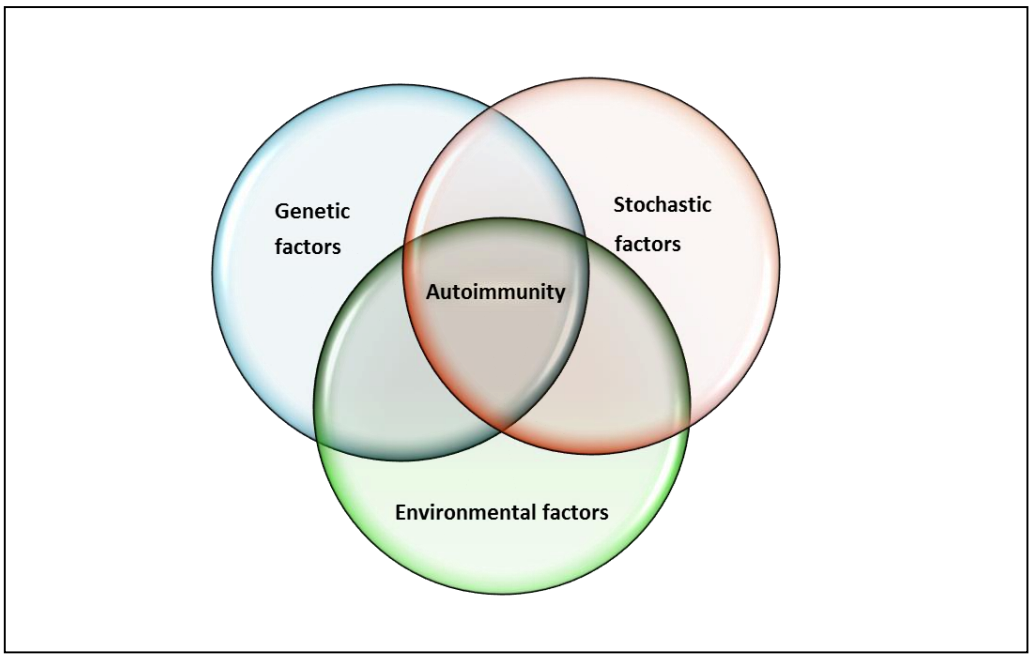


Figure 2.2 Multiple facets that lead to the precipitation of autoimmunity. The response elicited by the innate and adaptive arms of the immune system of a genetically predisposed individual to an environmental pathogen, in association with defects in immunoregulatory mechanisms and (or) stochastic event(s), can lead to the development of an autoimmune disease. The importance of each component represented in this Venn diagram may vary between individuals and diseases. However, it appears that the precipitation of autoimmunity requires the convergence of all these components. (modified after Ermann and Fathman, 2001).

Though several genetic risk factors have been identified that influence the susceptibility for T1D, progress in understanding its pathogenesis has been complicated by the individual and synergistic involvement of a large number (> 30) of predisposing genes (Cooper et al., 2008; Hattori et al., 1986; Rainbow et al., 2008; Todd et al., 1987; Wicker et al., 1995). These include the major histocompatibility complex (MHC) class II loci as well as non-MHC loci. Of them, the HLA genes, DR4-DR8 and DR3-DQ2, contribute the greatest risk for autoimmune diabetes since these haplotypes have been found in 90% of children diagnosed with the disease (Devendra and Eisenbarth, 2003). The variable number tandem repeat (VNTR) region of the insulin gene located on chromosome 11 is another major genetic susceptibility factor, with shorter forms of a VNTR on the insulin gene promoter are associated with disease, while longer forms confer protection (Bell et al., 1984; Bennett et al., 1995). The cytotoxic T lymphocyte antigen 4 (CTLA-4) and lymphoid tyrosine phosphatase (LYP) genes, which control the negative regulation of T-cell activation, are implicated in susceptibility to autoimmune diabetes (Smyth et al., 2004). Some rare forms of autoimmunity like Autoimmune Polyendocrine Syndrome (APS1) and X-linked polyendocrinopathy immunodeficiency and diarrhoea-syndrome (XPID) are monogenic, caused by defects in the autoimmune regulator gene (*AIRE1*) (Nagamine et al., 1997) and Fox-P3 (Bennett et al., 2000; Lyon et al., 1990) genes respectively. Analysis of disease concordance rates in monozygotic twins revealed a concordance of 15% in rheumatoid arthritis (Silman et al., 1993), 30-50% in

Type 1 Diabetes (Redondo et al., 1999) and 57% in lupus patients (Block et al., 1975; Winchester, 1992), suggesting that genetic predisposition is a major factor in auto immune disease (AID) susceptibility. Meanwhile the deviation from 100% concordance in these autoimmune diseases among monozygotic twins suggests a possibility of susceptibility genes interacting with potential environmental and/or stochastic factors. Investigators from the Barbara Davis Centre recently reported a near 95% concordance of autoantibodies among monozygotic twins from families at risk, yet the disease penetrance rate was ≈50%, suggesting a possible role of environmental factors in modulating T1D. The 15 times higher relative risk of disease for a sibling of T1D patient indicates a strong genetic contribution , but the etiological effect of an increasingly permissive environment is the most logical explanation for the rapid increase in T1D incidence in many parts of the western world since World War II (Patterson et al., 2001).

The dominant hypothesis in autoimmunity is suggestive of a unique environmental factor acting as a trigger for the autoimmune destruction of β-cells on a highly polygenic susceptibility background. However, no study so far has definitively identified the factor that might initiate this process, thus raising a possible role for stochastic factors in T1D development. Early life stochastic events could hold the answer to T1D-discordance in monozygotic twins (genetically identical individuals exposed to similar environments during childhood) of diabetic parents. Despite having identical genes and sharing the same

environment, the fact that less than 100% of NOD mice or MRL-*lpr* mice (inbred strains prone to diabetes or lupus) develop autoimmunity, with varied time of onset, severity and pathology, highlights the significance of stochastic factors in its pathogenesis (Eisenberg et al., 1987; Wen et al., 2008). Germ-free NOD mice or BB/W rats had higher diabetes incidence than their peers housed in SPF conditions, however disease onset and progression in germ-free animals varied in time, similar to that of the SPF animals (Rossini et al., 1979; Wen et al., 2008), suggesting an important role for stochastic factors in T1D development.

2.3 Role of innate immunity in type 1 diabetes

Discovery of a direct association of islet-autoreactive T cells with β-cell destruction in T1D patients and the ability of diabetogenic T lymphocytes to adoptively transfer disease into naive recipients demonstrated that this immune cell population is crucial in disease development (Bendelac et al., 1987; Coppieters et al., 2012; Unger et al., 2012). However, the activation of diabetogenic T cells requires participation form the innate immune system, comprised of both cellular components including macrophages, DCs, NK cells, NKT cells, and their membrane bound or secreted factors including PRRs, TNF superfamily members, interferons (IFNs), chemokines etc. Defects of innate immune system play a major role in the pathogenesis of T1D both in man and in animal models of this disease. These include, deregulated activation and function of APCs (Jansen et al., 1995; Litherland et al., 1999), reduced numbers and functions of regulatory cells such as

NKT cells and FoxP3$^+$ T cells (Baxter et al., 1997; Brusko et al., 2005; Wilson et al., 1998) and reduced counts of circulating neutrophils (Valle et al., 2013). Recent genome-wide association studies have identified genes of the innate immune system to be associated with susceptibility to autoimmune diabetes. These include genes encoding MDA-5, that recognises RNA viruses (Liu et al., 2009; Nejentsev et al., 2009; Smyth et al., 2006), and DHCR7 and CYP2R1, genes involved in vitamin D metabolism (Bailey et al., 2007; Cooper et al., 2011) and the genes encoding TLR-7 and TLR-8, which triggers host response against enterovirus infections (Cooper et al., 2009b). These findings demonstrate an important role for innate immune system in the pathogenesis of T1D.

2.3.1 Macrophages and dendritic cells

Macrophages are one among the foremost immune cells to infiltrate pancreatic islets in NOD mice (Dahlen et al., 1998), where they secrete proinflammatory cytokines including IFN-γ, TNF-α and IL-1β that are cytotoxic to β-cells (Yoon et al., 1998). Macrophage depletion results in total protection against insulitis and diabetes (Jun et al., 1999), suggesting a major role for these cells in the initiation and progression of T1D. DCs are principal antigen presenting cells that have an ability to induce activation of a naïve T cells. Increased accumulation of DCs are observed in the earliest islet infiltrates in T1D patients as well as in NOD mice, and studies using NOD mice suggest them to be capable of priming diabetogenic T cells (Green and Flavell, 1999; Turley et al., 2003). Interestingly

DCs from pancreatic lymph nodes transferred to prediabetic NOD mice protected them from autoimmune diabetes (Clare-Salzler et al., 1992). Similar results were achieved when prediabetic NOD mice were treated with granulocyte-macrophage colony-stimulating factor (GM-CSF), which induced tolerogenic DCs (Gaudreau et al., 2007), suggesting that DCs could be pathogenic or on the contrary protective in T1D, depending on the location, timing and cell types to which antigens are being presented.

2.3.2 NK cells

NK cells are a key component of innate immune response against several pathogens and tumors (Biron et al., 1999). Evidence suggests both protective and pathogenic roles for NK cells in T1D. Impaired NK cell numbers and functions were observed in diabetes patients as well as in NOD mice (Herold et al., 1984; Rodacki et al., 2007), suggesting a protective role for NK cells in T1D. On the contrary, NK cells are implicated in the lysis of rat islet cells (MacKay et al., 1986), and the depletion of NK cells in NOD mice inhibited cyclophosphamide precipitated diabetes (Maruyama et al., 1991), suggesting a role for NK cells in autoimmune diabetes development. Despite their pathogenic role in T1D, protective functions of NK cells have been highlighted in several studies. NOD mice, which spontaneously develop diabetes, have impaired NK cell functions (Ogasawara et al., 2004), and NK cells of complete Freund's adjuvant (CFA)-

administered NOD mice produced INF-γ, which protected these mice from diabetes. These findings substantiate the protective role for NK cells in T1D.

2.3.3 NKT cells

CD1d-restricted NKT cells are important immunoregulators, primarily due to their ability to secrete IL-4. NKT cells play key roles in adaptive immunity and in the regulation of autoimmune responses. T1D in humans is associated with reduced NKT cell numbers and a defect in their capacity to secrete IL-4 (Wilson et al., 1998). Reduced frequency of NKT cells correlate with enhanced disease in NOD mice and in the BB rat, and a germ line deletion of *CD1d* exacerbated the disease in NOD mice (Jordan et al., 2007; Shi et al., 2001). α-GalCer-induced activation of NKT cells protected NOD mice against autoimmune diabetes. The adoptive transfer of NKT cell into NOD mice achieved similar results (Sharif et al., 2001). These findings demonstrate a protective role for NKT cells in T1D.

2.3.4 Toll-like receptors

TLRs are type I transmembrane receptors found on the plasma membrane or in endosomal compartments. They are part of a family of PRRs that play a major role in detecting microbes and microbial constituents. Depending on the microbe, signalling through TLRs trigger an array of immune responses, including, production of inflammatory cytokines such as IL-6, IL-12, IL-18, IFNα/β, IFN-γ and TNF, the stimulation of chemokine-production from various tissue cells and the

priming of DCs to upregulate MHC I and II and costimulatory molecules that aids T and B cell activation. Distinct TLR signalling triggered by specific pathogen associated molecular patterns (PAMPs) can affect autoimmune diabetes. Results from studies on NOD mice carrying mutant TLR alleles indicate that while some infectious organisms initiate or exacerbate autoimmune β-cell destruction, others inhibit this destructive process (summarised in Table 2.5).

TLRs	Intervention in NOD mice	Role /Effects of TLR activation on diabetes	References
MyD88 (Chr 9)	NOD.*MyD88*$^{-/-}$	Did not develop T1D in SPF conditions, but developed the disease when kept germ-free; upregulation of CD25 and proliferation of $CD4^{+}CD25^{+}$ cells were inhibited.	(Wen et al., 2008)
	NOD.*Myd88* $^{-/-}$*.aire* $^{-/-}$ (Aire-deficient mouse is a model of human APECED, a multiorgan autoimmune disorder)	Severe infiltration in various susceptible organs than that of the NOD.*Myd88* $^{+/-}$*.aire* $^{-/-}$ controls.	(Gray et al., 2007)
	NOD.*IL1R*$^{-/-}$ (TLRs belong to the IL-1R/TLR superfamily, which include receptors for the pro-inflammatory cytokines IL-1 and IL-18; All members of TLR/IL-1R superfamily contain TIR domain and signals through MyD88, except TLR3)	Slowed the progression but did not prevent diabetes; did not affect diabetes in 8.3 TCR transgenic mice, but prolonged its progression in BDC2.5 TCR transgenic mice.	(Thomas et al., 2004)
	NOD/Lt R_X OM-85 (bacterial extract	Diabetes incidence halved, probably due to signalling through MyD88, TLR4/2.	(Alyanakian et al., 2006)
	CFA treated NOD.*MyD88*$^{-/-}$ hepatocytes	NOD NKT cell activation by CFA is independent of MyD88 signalling	(Lee et al., 2011)

Contd.

TLRs	Intervention in NOD mice	Role /Effects of TLR activation on diabetes	References
TLR1 (Chr 5)	NOD.C3H 6.VIII (diabetes-resistant congenic strain)	Constitutive and specific down-regulation of *Tlr1* gene	(Vallois et al., 2007)
TLR2 (Chr 3)	NOD.*TLR2*$^{-/-}$	Reduced the incidence of diabetes by 50% compared to WT Dispensable for T1D development; upregulation of CD25 and proliferation of $CD4^{+}CD25^{+}$ cells were inhibited.	(Kim et al., 2007) (Wen et al., 2008)
	NOD/Lt R_X zymosan (fungal cell wall component that interacts with TLR2)	Diabetes incidence reduced by about 50% compared to WT Signalling through TLR2 reduced diabetes incidence.	(Karumuthil-Melethil et al., 2008)
TLR3 (Chr 8)	NOD.*TLR3*$^{-/-}$	Dispensable for T1D development. Diabetes incidence in $TLR3^{-/-}$ mice not different from $TLR3^{+/-}$ littermates or wild type mice.	(Wen et al., 2008) (Wong et al., 2008)
	NOD/Lt R_X poly(I:C) (ds-RNA mimic and TLR3 agonist)	Diabetes precipitated in half of mice within a week of treatment.	(Peng et al., 2006)
TLR4 (Chr 4)	NOD.*TLR4*$^{-/-}$ NOD/Lt R_X (oral) LPS (ligand for TLR4)	Dispensable for T1D development. Accelerated development of diabetes and of body weight, regulates inflammatory functions of adipocytes in T1D progression.	(Wen et al., 2008) (Gulden et al., 2009)
		Halved the incidence of spontaneous diabetes	(Sai and Rivereau, 1996)
	NOD/Lt R_X LPS activated B cells	80% reduction in spontaneous diabetes	(Tian et al., 2001)

Contd.

TLRs	Intervention in NOD mice	Role /Effects of TLR activation on diabetes	References
TLR5 (Chr 1)	-	-	-
TLR6 (Chr 5)	-	-	-
TLR7/8 (Chr X)	9 week old NOD/Lt R_X Coxsackie virus B3 or lymphocytic choriomeningitis virus (LCMV)	Acute infection delayed onset and reduced diabetes incidence (probably a result of signalling through TLR7/8).	(Filippi et al., 2009)
TLR9(Chr 9)	NOD.TLR9$^{-/-}$	Delayed onset of diabetes, lower levels of IFN-alpha in PLNs and decreased frequencies of plasmacytoid DCs and diabetogenic CD8+ T cells compared to NOD.	(Zhang et al., 2010)
	NOD.*TLR9*$^{-/-}$	Reduced incidence of diabetes by about one third compared to their heterozygous littermates.	(Wong et al., 2008)
	NOD/Lt R_X 100μg CpG DNA (TLR9 ligand)	Diabetes incidence was halved.	(Quintana et al., 2000)
TLR10	-	-	-
TLR11 (Chr 14)	-	-	-
TLR12 (Chr 4)	-	-	-
TLR13 (Chr X)	-	-	

Table 2.5 Role of TLRs in mediating environmental influences on T1D. NOD mice carrying mutant TLR alleles were used to determine their role in mediating environmental influence on diabetes.

2.3.5 Cytokines and chemokines

Insulitic infiltrates including monocytes, macrophages, dendritic cells and NK cells produce cytokines such as IL-1, TNF-α and IFN-γ, which are cytotoxic to β-cells. IL-1 is selectively cytotoxic to rodent and human β-cells *in vitro* (Mathis et al., 2001), and NOD mice lacking the IL-1 receptor expression had a significantly delayed diabetes onset (Thomas et al., 2004). Animal experiments suggest that IL-1 induces FAS upregulation on β-cells, marking them for lysis by autoreactive cytotoxic T-lymphocytes (Amrani et al., 2000). An important effector of T1D pathogenesis, TNF-α promotes leukocyte homing, cell adhesion (Yagi et al., 1995), upregulation of MHC class I and MHC class II within pancreatic islets (David-Watine et al., 1990), and activation of T cells and APCs. Additional to being directly cytotoxic to β-cells, this cytokine also assists their FAS-dependent destruction (Varanasi et al., 2012). Results of studies indicating the association of serum TNF-α level with severity of T1D suggests that it is a crucial cytokine in the development of diabetes and associated complications (Argiles et al., 1994; Gustavsson et al., 2008; Schram et al., 2005). IFN-γ, a proinflammatory cytokine, though not detrimental to β-cells by itself, contributes towards β-cell apoptosis in association with IL-1β and TNF. A targeted mutation of IFN-γ gene in NOD mice had little effect on insulitis and spontaneous diabetes (Hultgren et al., 1996). IFN-γ is crucial for β-cell-specific MHC-I upregulation (Thomas et al., 1998) and iNOS proteins (Eizirik et al., 1996).

Activation of TLRs expressed on human and mouse islets mediate the upregulation of islet chemokines (Giarratana et al., 2004). Chemokines are central mediators of cellular trafficking and are implicated in diabetes development both in NOD mouse and in man. High levels of chemotactic cytokines including CCL3, CCL4 and CXCL10/IP-10 were observed in the sera of new onset T1D patients (Eizirik et al., 2009). Pancreatic islets under distress produce IP-10 that induces T cells to home in on islet cells, leading to insulitis and β-cell destruction (Roep et al., 2010). IP-10 neutralization in new onset diabetes inhibited β-cell destruction (Shigihara et al., 2005), suggesting a critical role for this chemokine in the autoimmune process. MIP-1α and MCP-1, chemokines secreted by autoreactive Th1 cells, play a role in the development of insulitis (Bradley et al., 1999). Protein mediated blockade of CCR5, receptor for the chemokine RANTES/CCL5 (Regulated on Activation, Normal T cell Expressed and Secreted) reduced disease in NOD mice (Carvalho-Pinto et al., 2004), suggesting a contributory role for it in T1D development. However, among all chemokines, IP-10 appears to be the most potent and probably the best-studied chemoattractant in the field of T1D, with several studies reporting islet-associated IP-10 expression in recent-onset as well as in advanced stages of T1D (Roep et al., 2010; Tanaka et al., 2009; Uno et al., 2010).

2.4 TNF superfamily ligands in innate immunity

Tumor necrosis factor superfamily ligands (TNFSFL) represent a family of type II transmembrane molecules that mediate vital biological functions including regulation of immune responses, inflammation, antiviral and anticancer defence (Beutler and van Huffel, 1994; Smith et al., 1994). Programmed cell death is a critical process in maintaining homeostasis in any cellular environment and it ensures immune responsiveness without incurring an autoimmune reaction. Biological elimination of damaged or unwanted cells is mainly achieved by apoptosis. Tumor necrosis factor superfamily ligands, FAS ligand (FASL) and TNF-related apoptosis-inducing ligand (TRAIL), induces apoptosis of target cells by binding to and ligating their complimentary TNF superfamily receptors (TNFSFR), FAS and DR4/5 respectively (Figure 2.3). Expressed predominantly by activated immune cells, TNFSFL are widely distributed through the immune system, suggesting an essential physiological role in innate immunity. NK cells, DCs and macrophages, effector cells of the innate immune system express TNFSFL on their plasma membrane, by which they mediate contact-dependent non-secretory apoptotic activity against a wide range of targets including autoreactive cells and cancer cells (Falschlehner et al., 2009; Herbeuval et al., 2003; Nielsen et al., 2012). TNFSFL have also been shown to mediate cross talk between NK cells and DCs, leading to the polarisation and amplification of Th1 immune mechanisms that results in heightened innate and adaptive immune functions (Vujanovic, 2011).

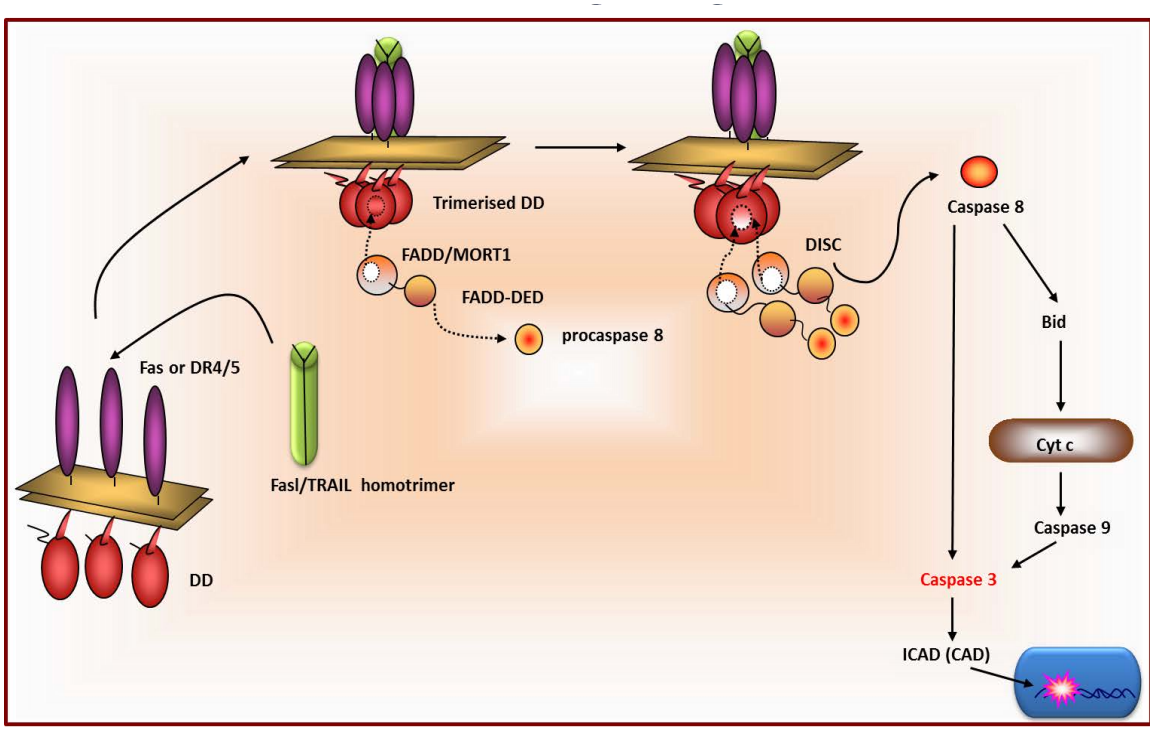


Figure 2.3 TNFSFL-signalling. TRAIL (Apo-2 Ligand/CD253/*Tnfsf10*) and FAS ligand (Apo-1 Ligand/CD95 Ligand/*Tnfsf6*) , type II transmembrane proteins of the TNF superfamily, on binding to its complimentary cell-surface type I-membrane protein receptors, death receptor 4 (DR4/TRAILR1/*Tnfrsf10A*) or 5 (DR5/TRAILR2/*Tnfrsf10B*) and FAS (Apo-1/CD95/*Tnfrsf6*) respectively, triggers apoptosis of target cells (Nagata and Golstein, 1995; Pan et al., 1997; Walczak et al., 1997). Trimeric TNFSFL binds to its complimentary receptors triggering its trimerization, which induces recruitment and activation of the common death signalling molecules FADD/MORT1. Caspase-8 is then recruited to FADD/MORT1 through binding of each death effector domains (DED), which in turn may induce self-activation of the protease domain and eventually leads cell to apoptosis.

2.4.1 TNFSFL in thymic negative selection

Autoimmune disease results from a breakdown of immunological tolerance to self-antigens. Maintenance of self-tolerance is achieved by a number of mechanisms including; thymic negative selection, suppression of autoreactive lymphocytes and functional inactivation (anergy). Daily, a large numbers of precursor cells migrate into thymus, where they are educated to discriminate between self and non-self. Apoptotic mechanisms shape the T cell repertoire by regulating thymocyte development and by eliminating potentially-autoreactive immature lymphoid population. Death ligands of TNF superfamily appear to be major contributors in this intrathymic negative selection process.

The influence of TRAIL on autoimmunity was initially described in two reports that suggested it to be critical for negative selection of autoreactive thymocytes (Corazza et al., 2004; Lamhamedi-Cherradi et al., 2003b). However, Simon and colleagues contested TRAIL's role in in antigen-induced deletion of thymocytes. They reported that anti-CD3 or superantigen-induced thymocyte cell death in human thymic organ cultures were uninhibited after the soluble blocking of TRAILR2-Fc. Similarly, Antibody mediated TRAIL receptor blockade was unable to prevent *in vivo* self-peptide induced deletion in TCR-transgenic mice (Simon et al., 2001). Moreover, transgenic expression of CrmA or a dominant negative mutant FADD protein, which inhibit TRAIL induced apoptosis (Suliman et al., 2001), did

not affect thymocyte elimination during negative selection (Walsh et al., 1998), and in some cases actually enhanced the deletion of autoreactive thymocytes (Newton et al., 1998). Using mice bearing a targeted deletion of TRAIL, or by utilising neutralizing anti-TRAIL mAb, Cretney and colleagues demonstrated a limited role for TRAIL in thymic negative selection (Cretney et al., 2003). Diehl and colleagues reported normal negative selection in targeted mutant $DR5^{-/-}$ mice, a finding consistent with the hypothesis that TRAIL-receptor signalling is not required for the selection process (Diehl et al., 2004).

Semi-mature (HSA^{hi} $CD4^{+}$ $CD8^{-}$) medullary thymocytes expressing non-functional or strongly autoreactive TCR specificities undergo Fas (CD95/Apo1) mediated clonal elimination. Negative selection of HSA^{hi} $CD4^{+}$ $CD8^{-}$ T cells in thymus is FAS independent at low doses of antigen but FAS dependent at high doses. Kishimoto and colleagues (Kishimoto et al., 1998) by subjecting purified $\alpha\beta TCR^{hi}$ semimature HSA^{hi} $CD4^{+}$ $CD8^{-}$ thymocytes, from normal adult B6 and FAS deficient $B6^{-lpr/lpr}$ mice, to anti-TCR monoclonal antibody (mAb), demonstrated that thymocyte apoptosis is partly FAS-dependent and is influenced by TCR ligation. It was observed that TCR/CD28 mediated apoptosis of FAS^{+} and FAS-deficient cells were identical when the cells were exposed to a fixed high concentration of plate bound anti-CD28 mAb and low-to-intermediate concentrations of plate bound anti-TCR mAb. In contrast, strong TCR/CD28 ligation failed to induce apoptosis of FAS-deficient cells alone, indicating that apoptosis is FAS-independent with weak-to-moderate TCR ligation

but becomes highly FAS-dependent with strong ligation. The authors closely paralleled the above *in vitro* findings in a series of *in vivo* experiments. On injecting a low-to-intermediate dose of soluble super antigen staphylococcal enterotoxin B (SEB) or anti-TCR mAb into normal C3H mice and FAS-deficient MRL$^{lpr/lpr}$ mice, a marked depletion of semimature HSA^{hi} CD4^{+} CD8^{-} medullary thymocytes was observed in neo-natal (day 4 old) FAS^{+} and FAS- deficient mice. In contrast, a higher dose caused the deletion of HSA^{hi} SP cells only in FAS^{+} and not in FAS-deficient mice. Similar results were obtained on injecting various doses of ovalbumin (ova) 323-339 peptides into FAS^{+} D011 TCR transgenic mice (having TCR specificity for ova 323-339, presented by IA^{d}) and FAS-deficient D011$^{lpr/lpr}$ mice (Kishimoto et al., 1998), thus reiterating the above observations that deletion becomes FAS-dependent only with high antigen concentrations.

Impaired deletion of autoreactive thymocytes has been associated with susceptibility to autoimmune diabetes in the NOD mouse (Kishimoto and Sprent, 2001; Lesage et al., 2002). Semimature CD4^{+}8^{-} SP thymocytes from NOD, in comparison to similar cells from normal and other autoimmune disease prone mice, are relatively resistant to *in vitro* apoptosis when their TCRs are cross-linked with varying doses of anti-TCR antibody (Kishimoto and Sprent, 2001). However, Villunger and colleagues disputed this finding by demonstrating an efficient *in vitro* TCR-CD3 ligation mediated apoptosis of semimature CD4^{+}8^{-} SP thymocytes from NOD mice. By treating CD4^{+}8^{-}HSA^{hi} cells from normal, FAS-deficient *lpr*, and mice expressing a dominant negative mutant of FADD (FADD-DN) with anti-CD3

plus anti-CD28 antibodies, they furthered their point that FAS, and FADD-transduced death receptor signalling in general, is dispensable for TCR induced apoptosis of semimature thymocytes *in vitro* (Villunger et al., 2003). They also reported that the loss of Bim, a pro apoptotic molecule, but not a deficiency in FAS, inhibited TCR induced apoptosis of semimature thymocytes *in vivo* (Villunger et al., 2004), indicating a limited role for FAS in the clonal elimination of autoreactive cells in the thymus. Lesage and colleagues analysed thymic deletion *in vivo*, tracing differentiation of T cells bearing a high affinity TCR for peptide 46–61 of hen egg lysozyme (HEL) bound to I-A^k in TCR and insulin promoter:HEL double transgenic mice backcrossed to $H2^k$ congenic NOD or B10 strains (Lesage et al., 2002). These cells were deleted during the transition between DP and SP cells in the thymus of autoimmune resistant B10.Br strain mice, but not in the NOD.$H2^k$ strain where the T cells escaped to the periphery and progression to diabetes ensued. The NOD defect in thymic deletion was comparable in magnitude to that caused by deficiency of AIRE in the same model (Liston et al., 2003). Taken together, data in the literature illustrate contradictory roles for TNF superfamily ligands TRAIL and FASL with respect to thymic selection.

2.4.2 TNFSFL in peripheral tolerance

The regulation of cell proliferation and the elimination of autoreactive cells in the lymphoid system both occur through the induction of apoptosis. Ashwell and colleagues reported activation-induced cell death (AICD) in murine T cell

hybridomas (Ashwell et al., 1987). Even as TRAIL's role in thymic deletion is contradictory, several studies have implicated this death ligand in effecting peripheral T cell tolerance. Functional TRAIL-R is expressed on activated T cells in human and mouse, but not on resting T cells (Herbeuval et al., 2005; Martinez-Lorenzo et al., 1998; Zhang et al., 2003), suggesting TRAIL mediated T cell regulation. The adoptive transfer of TRAIL-overexpressing murine T cells induced apoptosis of alloreactive T cells, in a DR5-dependent manner (Ghosh et al., 2013). In a tolerance model developed by Griffith and colleagues, TRAIL-mediated-apoptosis inhibited the expansion of islet-antigen-reactive $CD4^+$ T cells (Griffith et al., 2007). All these findings are consistent with a role for TRAIL in the regulation of cell cycle progression and in the elimination of autoreactive cells.

Stimulation of T cell hybridomas with antigens, mitogens or TCR/CD3 specific antibodies triggered growth arrest, functional activation (secretion of IL-2) and apoptosis. Mature human T cells were reported to acquire sensitivity to agonistic anti-CD95 mAb only after a prolonged *in vitro* activation (Klas et al., 1993; Owen-Schaub et al., 1992), suggesting that T cell receptor (TCR) mediated activation sensitises T cells to CD95 mediated AICD. An *In vivo* study showed that among lupus-prone NZB, (NZB x NZW)F1, BXSB and $MRL^{lpr/lpr}$ mice, only FAS- deficient *lpr* mice had an impaired deletion of $CD8^+V\beta8^+$ cells even following a high dose administration of the superantigen Staphylococcal enterotoxin B (Scott et al., 1993). Russell and colleagues used the superantigen staphylococcal enterotoxin

(SEA) *in vitro* to test the involvement of CD95 in AICD. Mature T cells from CD95 deficient *lpr/lpr* mice exhibited a normal activation pattern, but were resistant to apoptosis induced by a subsequent high dose antigenic challenge, indicating that CD95 is essential for AICD. By stimulating wild type and *lpr/lpr* T cells with various concentration of SEA on antigen presenting cells (APC) and plastic immobilised anti-CD3 antibodies in the absence of APCs, they demonstrated that peripheral cells reactivated through TCR/CD3 complex, underwent FAS-mediated AICD in an APC-independent manner (Russell et al., 1993). Russell and Wang studied CD95L-deficient *gld/gld* mice adopting a similar methodology as above and confirmed a role of CD95L in AICD (Russell and Wang, 1993). High concentrations of immobilized anti-CD3 antibody induced both functional anergy and apoptosis in wild-type T cell *in vitro*, while *lpr* T cell lines were relatively resistant to apoptosis (Bossu et al., 1993). Singer and Abbas compared the T cell response in TCR transgenic (specific for the 81–104 peptide of pigeon cytochrome c) wild type and *lpr* mice. On chronic exposure to the reactive antigen *in vivo*, in contrast to wild-type, the peripheral FAS-defective *lpr* T cells were persistent, while similar elimination of thymocytes occurred in both strains, indicating that the role of FAS is mainly restricted to the periphery (Singer and Abbas, 1994).

In vitro experiments by Brunner and colleagues (Brunner et al., 1995) as well as by Ju and co-workers (Ju et al., 1995) implicated autocrine stimulation of CD95 in AICD of hybridoma T cells, while Dhein and colleagues demonstrated this in Jurkat

T leukaemia cells and non-transformed, preactivated T cells (Dhein et al., 1995). All three studies showed that TCR stimulation induces CD95 and CD95L, and that apoptosis can be inhibited by either down regulating CD95 using glucocorticoids (Ju et al., 1995) or by blocking CD95 with non-stimulatory FAS-specific antibody fragments or a chimeric CD95-immunoglobulin fusion protein (Brunner et al., 1995), or else by inhibiting the induced expression of CD95L using cyclosporin A (Dhein et al., 1995). MacDonald and colleagues showed that normal T cells could suppress the CD95L-defective *gld/gld* phenotype in chimeric mice (MacDonald et al., 1995), indicating that FAS not only mediates apoptosis in a cell-autonomous way but can also trigger apoptosis of syngeneic, activated neighbouring lymphocytes (Vignaux and Golstein, 1994). These findings support a role for FAS in a regulatory mechanism, which controls the expansion of activated T cell populations.

To demonstrate that activated human peripheral blood B cells acquire sensitivity to FAS-mediated apoptosis, SCID mice were reconstituted with human peripheral blood mononuclear cells (PBMC). On an adjuvant-free tetanus toxoid or diphtheria toxoid challenge in the presence of an agonistic anti-CD95 mAb, these PBMCs showed antigen specific immunosuppression (Daniel and Krammer, 1994), suggesting an involvement of FAS in the regulation of humoral immune response. Rothstein and colleagues in an *in vitro* study reported CD40-ligand-stimulated

naïve B cells to be extremely sensitive to FAS-mediated apoptosis, while, anti-IgM-stimulated B cells were resistant, regardless of the presence of CD40L (CD154) (Rothstein et al., 1995). Specific FAS-mediated elimination of $CD154^+$ $CD4^+$ T cell-activated bystander B cells suggests a role for FAS in the selection and expansion of antigen-activated B cells. Rathmell and co-workers reported that FAS-FASL system mediated the elimination of self-reactive B cells. They studied the interaction of transgene encoded autoantigen hen egg lysozyme (HEL)-recognising B and $CD4^+$Tcells in an *in vivo* model. The authors observed that HEL-specific $CD4^+$Tcell eliminated anergic B cells that previously encountered antigen, *via* FAS (Rathmell et al., 1995). These reports highlight the crucial role for TNF superfamily ligands TRAIL and FASL in instituting self-tolerance in the T cell as well as B-cell compartment.

2.5 TNFSFL in autoimmunity and type 1 diabetes

Mice with targeted gene deletion of TRAIL and mice given anti-TRAIL monoclonal antibody (sDR5) were more susceptible to autoimmune disease, including, arthritis and experimental autoimmune encephalomyelitis (EAE) (Cretney et al., 2005; Hilliard et al., 2001; Lamhamedi-Cherradi et al., 2003b; Song et al., 2000), suggesting a role for TRAIL in suppressing these autoimmune conditions. In these studies, TRAIL appeared as a negative regulator of the innate arm of the immune system. Consistent with this hypothesis, mice deficient in TRAIL signalling exhibited increased levels of IL-12, IFN-α and IFN-γ (Diehl et al.,

2004). Contrary to findings from studies that suggest a suppressive role for TRAIL in T cell-mediated autoimmune disease, evidence of a detrimental role for this molecule in autoimmunity has emerged. TRAIL blockade within the central nervous system suppressed EAE by inhibiting brain cell apoptosis (Aktas et al., 2005). Furthermore, TRAIL-expressing T cells exacerbated parent into F1 graft-versus-host disease by positively regulating $CD4^+$ T cell numbers, thereby sustaining T cell help for B cells, and negatively regulating perforin-mediated $CD8^+$ cytotoxic T lymphocyte-killing that could have potentially eliminated activated autoreactive B cells (Rus et al., 2007). Absence of TRAIL reduced allergic airway disease in mice (Weckmann et al., 2007) by mediating the induction of CCL20, which recruits IL-4 secreting cells. CCL20 and IL-4 secreting cells play a role in suppressing autoimmune diseases like T1D and EAE. These findings illustrate a pleiotropic nature for TRAIL in the innate immune system.

Apoptotic signalling by soluble or membrane-bound cell death ligands (FASL and TRAIL) could play a role in some of the pathways that mediate pancreatic β-cell death in T1D patients and in NOD mice (Chervonsky et al., 1997; Cheung et al., 2005). However, the expression of TRAIL and its receptors on human islets is currently disputed. Immunohistochemical analysis by Sanlioglu and colleagues indicated that human islets primarily expressed TRAIL and its two decoy receptors but not the death receptors DR4/5. BioGPS (http://biogps.gnf.org/) the gene annotation portal of the Novartis Research Foundation reports no message for

DR4/5 or decoy receptor 1, but expression of TRAIL in human islets. Partly contrasting with these findings Ou and colleagues reported the expression of DR1-4 and/or TRAIL on human islet cells by RT-PCR and flow cytometry (Ou et al., 2002), however, their experiment lacked adequate controls. They also demonstrated that islet cells are resistant to TRAIL-induced cytotoxicity. Ou and colleagues examined this by co-culturing recombinant human TRAIL with Cr-labelled β-cell lines and human primary islet cells. While TRAIL induced killing of the cell lines CM and HP62 at 2.5ng/ml within 4 hours, the primary islet-cells of only one of five donors were apoptosed by concentrations as high as 80ug/ml. CDNA array analysis indicated that TRAIL expression is upregulated in pancreatic islets during the development of T1D in NOD mice (Mi et al., 2003). Mi and colleagues further demonstrated that TRAIL-signalling was unable to induce apoptosis in freshly isolated human pancreatic islets. TRAIL-signalling did it have any significant cytotoxic effect on pancreatic β-cell cultures (Tekmen et al., 2007), suggesting that most normal islet cells are resistant to TRAIL-mediated cytotoxicity. The transgenic expression of TNF superfamily members on pancreatic islets exacerbated insulitis and/or diabetes in mouse models (Chervonsky et al., 1997; Higuchi et al., 1992; Miwa et al., 1998) . On the contrary, an enhanced incidence of diabetes and aggravated islet cell destruction was reported in TRAIL-deficient C57BL/6 mice treated with multiple low-dose streptozotocin (Lamhamedi-Cherradi et al., 2003b). The blockade of TRAIL signalling using a soluble receptor (sDR5) exacerbated the onset of streptozotocin-

induced diabetes (Lamhamedi-Cherradi et al., 2003a) and enhanced the adoptive transfer of the disease into NOD.*scid* mice by diabetogenic splenocytes from NOD mice (Mi et al., 2003). Taken together, these findings suggest a β-cell protective role for TRAIL.

Apoptosis of β-cells is a feature of autoimmune diabetes (Kurrer et al., 1997), and immunohistochemical analysis has revealed FAS expression on both murine and human β-cells (Chervonsky et al., 1997; Stassi et al., 1997). Pancreatic biopsy of patients with newly diagnosed T1D revealed constitutive expression of FAS on their β-cells, while the islet-infiltrating cells was found to express FASL (Loweth et al., 1998; Moriwaki et al., 1999). Stassi and colleagues observed extensive apoptosis among pancreatic β-cells located in proximity to FASL-expressing T cell infiltrates of the islets by immunohistochemistry (Stassi et al., 1997). These observations suggests an important role for FAS-FASL signalling in β-cell apoptosis. Development of autoimmune diabetes in NOD mice is characterised by two checkpoints. Firstly, 3-4 week old NOD mice spontaneously develop lymphocytic infiltrates in islets of Langerhans and secondly, by about 12 weeks of age the insulin production drops due to FAS-FASL and perforin-granzyme mediated specific destruction of β-cells, resulting in diabetes (Andre et al., 1996; Chervonsky et al., 1997; Itoh et al., 1997; Kagi et al., 1996; Kagi et al., 1997). Findings in NOD mice that β-cell expression of FAS correlates with the second checkpoint of autoimmune diabetes (Suarez-Pinzon et al., 1999), suggested a

causal role of FAS in the transformation of benign infiltration into a detrimental destruction of islets. Administration of neutralising anti-FASL monoclonal antibody significantly inhibited $CD4^+$ T cell adoptive transfer of diabetes (Nakayama et al., 2002), suggesting a significant role for FAS-FASL interaction in T cell mediated insulitis.

Initial indication of the role of FAS in β-cell damage came from studies on FAS deficient $NOD^{lpr/lpr}$ mice (Chervonsky et al., 1997; Itoh et al., 1997). These mice neither spontaneously developed diabetes or insulitis nor did the transfer of autoreactive $CD8^+$ T cell clones or diabetogenic splenocytes from NOD mice initiate its induction. Adoptive transfer of a diabetogenic islet specific perforin positive $CD8^+$ T cell clone (TGNF-H3) into FAS deficient $NOD^{lpr/lpr}$ mice developed neither diabetes nor insulitis, suggesting that FAS played a more important role than perforin in the induction of autoimmune diabetes (Chervonsky et al., 1997; Itoh et al., 1997; Wong et al., 1996). Studies by Kagi and colleagues on transgenic mice expressing glycoprotein of lymphocytic choriomeningitis virus in the β–cells of pancreas indicated that $CD8^+$ T cells require perforin to cause β-cell damage (Kagi et al., 1996). However, their later experiments revealed that although perforin-deficient mice developed severe insulitis, they had delayed onset and reduced incidence of diabetes, compared to wild type mice (Kagi et al., 1997). Adoptive transfer experiments by Amrani and colleagues using diabetogenic $CD8^+$ T cells bearing a T-cell receptor (8.3-TCR) that targets an immunodominant

peptide/H-2Kd complex on β-cells contested a role for perforin in β-cell damage. 8.3-cytotoxic T lymphocytes (CTLs) from perforin-deficient NOD mice were as diabetogenic as 8.3-CTLs from 8.3-NOD.*PO*$^{+/+}$ mice when adoptively transferred into immunodeficient RIP-B7.1–transgenic NOD.*scid* mice. They reported that perforin-deficient 8.3-TCR-transgenic NOD mice developed spontaneous diabetes with a frequency similar to that in their perforin-competent littermates. Further *in vitro* experiments by the authors revealed that while 8.3-CD8^{+} CTLs from both 8.3-NOD.*PO*$^{+/-}$ and 8.3-NOD.*PO*$^{-/-}$ mice killed NOD islet cells, they were unable to eliminate NOD.$^{lpr/lpr}$ islet cells, suggesting that β-cell damage occurred in a perforin independent and FAS-dependent manner (Amrani et al., 1999). In the same year Kreuwel and colleagues used a mouse model that had the influenza hemagglutinin (HA) transgenically expressed as a self-antigen exclusively on the β-cells, to directly compare both perforin-granzyme and FAS-FASL cytotoxic pathways in β-cell destruction. Islet destruction and diabetic induction subsequent to the introduction of HA-specific CTL (clone -4TCR) that was deficient in either FASL or perforin were compared. It was observed that FASL deficient effector cells (clone 4 TCR-*gld*) were comparable to normal effectors in their β-cell destruction ability, whereas perforin-deficient clone-4 TCR CTL were 30 fold less efficient in destroying islets and causing diabetes than normal CTL (Kreuwel et al., 1999), indicating that perforin is much more efficient in lysing β-cells than FAS in this model. These findings indicate that both FAS and perforin are able to inflict β-cell damage in a mutually exclusive manner.

Findings that reported defects in FAS-FASL system to provide protection against NOD diabetes lead to the presumption that FASL expression was detrimental for β-cell survival. However, the numerous immune abnormalities in NOD$^{lpr/lpr}$ mice were not accounted for in these studies. The absence of insulitis in this model would suggest the inability of NOD$^{lpr/lpr}$ T cells to enter the pancreas; thereby rendering this model unsuitable for the evaluation of FAS mediated β-cell destruction. Chervonsky and colleagues reported that FAS expression on islet cells of NOD mice (12 weeks or older) was upregulated after transfer of diabetogenic T cell clone, but they did not examine the FAS expression in the spontaneous NOD mouse model (Chervonsky et al., 1997). To test the validity of NOD$^{lpr/lpr}$ as a model for the evaluation of FAS mediated β-cell destruction Allison and Strasser transferred CFSE-labelled NOD spleen cells into irradiated and non-irradiated NOD$^{lpr/lpr}$ hosts, and found that the transferred cells were preferentially depleted in the hosts (Allison and Strasser, 1998). This could be explained by the fact that the *lpr* mutation in mice is associated with upregulation of FASL on CD4^{-} CD8^{-} B220^{+} α/β T cell population and these cells can kill FAS-expressing cell lines *in vitro* (Chu et al., 1995). Apostolou and colleagues suggested that FAS is dispensable for the induction of insulitis. The authors compared the onset and development of diabetes in a β-cell-specific *Fas* deficient model of autoimmune diabetes with that in mice that expressed FAS on β-cells (in both cases mice had a transgenic TCR specific for influenza hemagglutinin (HA) and they expressed HA under control of the rat insulin promoter. In this model, the elimination of *Fas* did

not cause a delay in the onset of diabetes but conversely accelerated the disease in β-cell-specific FAS deficient mice (Apostolou et al., 2003). Using chimeras of the *lpr* mutation and a mouse bearing diabetogenic $CD4^+$T cell associated transgenic TCR ($NOD^{lpr/lpr.BDC2.5}$), Vence and co-workers demonstrated that the modulation of diabetes in *lpr* mice was due to abnormalities in T cell function rather than inhibition of cytotoxicity to the β-cell. They observed that $NOD^{lpr/lpr.BDC2.5}$ mice have qualitatively and quantitatively less aggressive insulitis than $NOD^{BDC2.5}$ mice. *In vitro* proliferation assays indicated that $NOD^{lpr/lpr.BDC2.5}$ splenocytes proliferated less vigorously than those from control mice in the presence of islet extracts (Vence et al., 2004). RNase protection assay by Kim and colleagues showed that $NOD^{lpr/lpr}$ lymphocytes constitutively expressed high levels of FASL. To prove that abnormal $FASL^+$ lymphocytes from $NOD^{lpr/lpr}$ are responsible for the inhibition of diabetes transfer, they co-transferred splenocytes from diabetic NOD mice and $FASL^+$ lymphoid cells from $NOD^{lpr/lpr}$ or $NOD^{lpr/+}$ mice to sub lethally irradiated NOD mice. It was observed that none of the nine NOD mice that received lymphocytes from $NOD^{lpr/lpr}$ developed diabetes, while 75% of NOD recipients of $NOD^{lpr/+}$ lymphocytes became diabetic. Furthermore, administration of anti FASL monoclonal antibodies reversed the inhibition of diabetes transfer (Kim et al., 2000). These findings suggest that diabetes protection in *Fas/FasL* impaired mice could be an epiphenomenon associated with their distorted immune system.

Su and colleagues reported that FAS deficient $NOD^{lpr/lpr}$ and FASL deficient

NOD$^{gld/gld}$ mice are resistant to the development of spontaneous diabetes. Interestingly in this study NOD$^{gld/gld}$ littermates heterozygous for the FASL mutation (NOD$^{gld/+}$), which lacked the abnormal DN T cell population and lymphadenopathy seen in NOD$^{lpr/lpr}$ and NOD$^{gld/gld}$ mice, also failed to develop diabetes. However, the manifestation of a non-invasive insulitis in these mice indicated the generation of an anti-islet response, providing evidence supporting a role for FASL-mediated apoptosis in the pathogenesis of autoimmune diabetes. Further, they generated a novel NOD$^{lpr/lpr\text{-}scid/scid}$ mouse model that lacked T and B cells and in which the FASL mediated lytic activity was abolished. Following adoptive transfer of splenic cells from diabetic NOD mice, NOD$^{lpr/lpr\text{-}scid/scid}$ mice had a reduced diabetes incidence when compared to their FAS-expressing NOD$^{scid/scid}$ littermates. The severity of disease was also significantly reduced in the FAS-deficient recipients. While around 90% destructive insulitis was observed in the NOD$^{scid/scid}$ recipients of diabetogenic NOD lymphocytes, the rate of cellular infiltration in the *lpr/scid* double mutant recipients was reduced by half (Su et al., 2000), suggesting a role for islet expressed FAS in the pathogenesis of the disease. Savinov and colleagues adoptively transferred splenic cells from diabetic NOD mice to NOD.*scid*, NOD$^{lpr/lpr\text{-}scid/scid}$, NOD$^{RIP\text{-}FasL.scid}$ (transgenic mice expressing FASL in β-cells) and NOD$^{RIP\text{-}FasL.lpr/lpr\text{-}scid/scid}$ mice (transgenic for FASL and lacking endogenous FASL-expressing lymphocytes). They reported that while all NOD.*scid* and NOD$^{RIP\text{-}FasL.scid}$ (FAS sufficient) mice became diabetic, only 70% of FASL expressing NOD$^{RIP\text{-}FasL.lpr/lpr\text{-}scid/scid}$ mice went diabetic. Moreover, the diabetes onset

in all of the FASL expressing NOD$^{RIP\text{-}FasLscid}$ was highly accelerated compared to NOD$^{RIP\text{-}FasL.lpr/lpr\text{-}scid/scid}$ (Savinov et al., 2003), indicating that lack of FAS signalling delayed the diabetes development and that β-cell killing in some measure was mediated by the ligation of the death ligand with its receptor. It also suggested that other mechanisms might also be participating in the killing of β-cells as FAS deficient mice became diabetic on adoptive transfer of effector cells. The transgenic expression in β-cells of a decoy receptor (DCR3) protective against FASL-induced cell death prevented autoimmune insulitis and prolonged transgenic islet-graft survival in NOD mice (Sung et al., 2004), indicating that the destruction of β-cells in IDDM could be mediated by FAS-FASL interactions. Allison and co-workers (2005), generated NOD mice that expressed a dominant negative form of FAS associated death domain (dnFADD) adaptor to block death receptor signalling in β-cells. While β-cells from the transgenic mice were resistant to FAS-mediated apoptosis *in vitro* and a reduced incidence of diabetes was observed in mice with higher levels of dnFADD expression (Allison et al., 2005), thereby suggesting that FAS played a role, although minor, in the pathogenesis of IDDM in this model.

Despite experiments that overcame the immune abnormalities of *lpr* recipient models and redefined the role of FAS in autoimmune diabetes, the basis for a lack of spontaneous insulitis and diabetes development in FAS deficient NOD$^{lpr/lpr}$ mice remained unresolved. Based on *in vitro* cytotoxicity assays using infiltrating cells

from pancreatic islets cells of NOD mice at early and late stages of insulitis on target NIT-1, BTC-3 or P815 cells, Qin and colleagues reported that perforin independent damage of β cells (FAS mediated) in early stages of insulitis switched to a perforin-dependent β-cell destruction at later stages of diabetes (Qin et al., 2004). This finding indicated an age-related change in the apoptotic pathways used by autoreactive CTLs in NOD mice. Based on this finding they proposed that the deficiency of FAS and FASL in *lpr/lpr* and *gld/gld* mice strains would prevent β cell apoptosis, if early autoreactive CTLs exclusively utilised FAS-FASL for β cell destruction. In the absence of this early FAS-mediated apoptosis, there would be neither antigen shedding nor consequently insulitis, thereby abrogating a perforin-dependent disease progression. The observations that NOD$^{lpr/lpr}$ and NOD$^{gld/gld}$ models did not develop diabetes (Itoh et al., 1997; Su et al., 2000) support this hypothesis. Also, consistent with the above finding is the fact that anti-FASL monoclonal antibody affects the development of diabetes most profoundly when administered to young NOD mice (Nakayama et al., 2002). Several studies have reported that incomplete triggering of $CD8^+$ T cells, as occurs through recognition of self-peptides that are antagonist or partial agonists, can result in FAS-mediated apoptosis but not the release of granule-associated enzymes (Brossart and Bevan, 1996; Cao et al., 1995; Esser et al., 1996). These data suggest that a weak triggering of $CD8^+$T cell TCRs could induce FAS-FASL to become the dominant pathway of cytotoxicity instead perforin-granzyme, which is consistent with the model proposed by Qin and colleagues (Qin et al., 2004).

Savinov and colleagues had reported that some essential stages of diabetes development might depend more on FAS mediated cytotoxicity than others and that interference with the FAS pathway early in the disease development may retard or prevent diabetes (Savinov et al., 2003). These findings put together could provide a unifying model for the discordant observations on the role of FAS-FASL and perforin-granzyme mediated cytotoxic pathway in the destruction of β-cells.

Even as majority of findings summarised so far in this section indicate a role for FAS-FASL system in pancreatic β-cell destruction, there is evidence that points to a protective role for this ligand in T1D induction. Signore and colleagues reported that under physiological conditions islet α-cells expressed FASL in both diabetes-resistant and diabetes-prone mice and that the β-cells were not adversely affected by the presence of FASL on islet α-cells, rather it shielded the FAS expressing β-cell from immune attack (Signore et al., 1997). Kim and colleagues studied the involvement of FAS in the development of diabetes by administering anti-FASL antibody and by observing FAS-deficient neonatal pancreas grafts from NOD$^{lpr/lpr}$ in diabetic NOD mice (to circumvent the immune abnormalities in *lpr* recipient). Anti-FASL antibody administration failed to inhibit diabetes in NOD mice after cyclophosphamide treatment or adoptive transfer of diabetogenic spleen cells, and FAS-deficient neonatal pancreata were destroyed by lymphocytic infiltration in diabetic NOD mice, thereby ruling out a major role for FAS in diabetes induction (Kim et al., 1999). They further demonstrated in an adoptive transfer model of

autoimmune diabetes, that rather than a β-cell effector function, the FAS-FASL system had an immune regulatory role (Kim et al., 2000). FASL expressing FoxP3^{+} regulatory T cells injected into NOD mice in the pre insulitis phase was reported to provide protection against diabetes-induction (Weber et al., 2006). Su and colleagues observed that insulitis observed in the *lpr/scid* double mutant recipients of diabetic NOD T cells contrasted with the complete absence of cellular infiltrates after adoptive transfer to irradiated NOD$^{lpr/lpr}$ hosts, indicating that autoreactive T cells might be sensitive to FASL-mediated elimination. NOD mice, prone to spontaneous diabetes have lower FASL expression compared to nondiabetes-prone BALB/c mice, owing to a mutation in *Fasl* (Kayagaki et al., 1997), suggesting a limited or even a protective role for this death ligand in autoimmune diabetes.

Timing of TNFSFL expression and the cells/tissue types in which they are expressed, could determine the effect (whether detrimental or protective) of these death ligands in autoimmune diabetes, and also explain the discordant findings in the literature, discussed above. Further investigation is warranted to unravel any potential β-cell protective roles for TRAIL and FASL, which could result in the development of treatment modalities for T1D management.

2.6 Mycobacterium bovis (BCG)

Bacillus Calmette-Guerin (BCG), a live attenuated strain of *Mycobacterium bovis* (which causes bovine tuberculosis) is used worldwide to prevent tuberculosis and leprosy. The current vaccine was developed by Calmette and Guérin, who continuously passaged a strain of *M. bovis* 230 times *in vitro*, which reduced its virulence while preserving the immunogenicity (Calmette and Guérin, 1911; Calmette and Guérin, 1920) . DNA studies by Baess and Mansa revealed that the human and bovine tubercle bacilli and BCG shared similar genome size and G:C content (Baess and Mansa, 1978). Mycobacteria have long been known to have a stimulatory effect on the cells of the immune system. BCG have been shown to enhance or contrarily, downregulate immune responses under experimental conditions (Chee and Bodurtha, 1974; Ishibashi et al., 1978). Knowledge of mycobacterial cell envelope-structure is imperative to understand its effects on host immune response. Several models have been proposed for the mycobacterial cell wall (McNeil and Brennan, 1991; Minnikin, 1991; Rastogi, 1991). The mycobacterial cell envelope is essentially composed of four layers (Figure 2.4). The first layer consists of the plasma membrane (PM) enclosing the cell's cytoplasm. It contains a permeable lipid bilayer with interacting membrane proteins (MP). The peptidoglycan (PG) / arabinogalactan (AG) moieties that make up the structural component of the cell wall is situated in the second layer, which is known as the electron dense (ED) layer by virtue of its staining properties when observed by transmission electron microscopy. The adjacent layer is electron

transparent (ET) comprising of mycolic acid and other complex lipids like lipoarabinomannan (LAM). The outermost layers (L1 and L2) vary according to the species, but are generally fibrillar in nature when observed by freeze fracture or negative staining (Barrow and Brennan, 1982; Draper, 1974; Rulong et al., 1991). The components of the outer layers of the mycobacterial cell envelope, especially glycopeptidolipids are crucial in the initial host interaction. They induce various host responses to help protect the organism from the detrimental effects of the phagolysosomal environment in the host macrophages where they reside during the infectious disease process (Bermudez, 1994; Tassell et al., 1992).

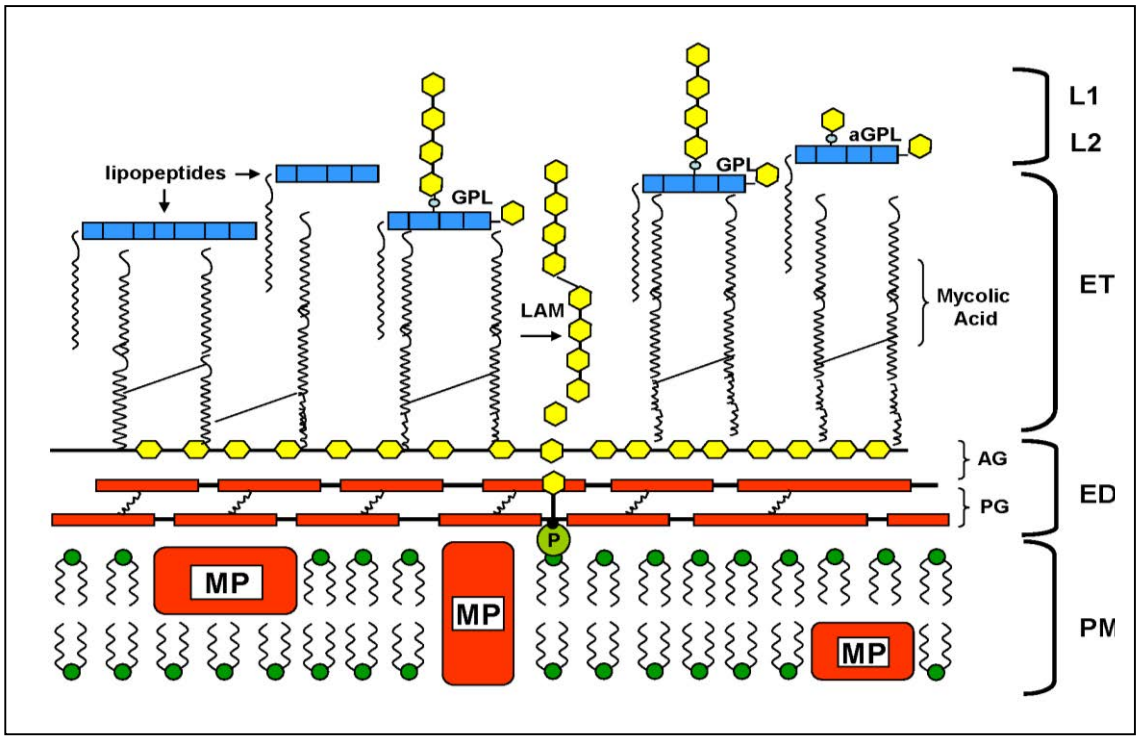


Figure 2.4 Model of mycobacterial cell envelope (not drawn to scale). Glycopeptidolipid (GPL), apolar GPL (aGPL), membrane proteins (MP), peptidoglycan (PG), arabinogalactan (AG), lipoarabinomannan (LAM), outermost layers of the wall (L1 & L2), electron transparent (ET), electron dense (ED), plasma membrane (PM).

BCG GPLs like phosphatidylinositol dimannosides, phosphatidylglycerol and cardiolipin, phosphatidylethanolamine, trehalose monomycolate, trehalose dimycolate (TDM), and mycoside B**,** traffic within the host macrophage and spread to uninfected bystander macrophages (Beatty et al., 2000; Rhoades et al., 2003). The GPLs, by virtue of their inert nature, persist in the host and accumulate within various sites of the reticuloendothelial system. A retrospective analysis of data on

AIDS patients who contracted mycobacterial infection revealed the involvement of the organism mainly in the lymph nodes (74%), spleen (74%) and liver (52%) (Abdel-Dayem et al., 1996).

Constituents of BCG bind to TLRs 1, 2, 4 and 9, type I transmembrane receptors found on the plasma membrane or in endosomal compartments of host immune cells. TLR-signalling activates macrophages and DCs to release pro-inflammatory cytokines including IL-12 and chemokines. This triggers the functional maturation of DCs, including the upregulation of CD80/86, which ligates with CD28 on T cells to initiate antigen-specific adaptive immune response and functional maturation of T cells. Purified mycobacterial cell-wall components have been shown to preferentially activate TLR2, and to a lesser extent, TLR4 (Heldwein et al., 2003). Prolonged signalling with a 19 KDa lipoprotein from Mycobacterium, which stimulates TLR2, inhibited IFN-γ production and MHC class II antigen-processing activity (Pai et al., 2004; Pennini et al., 2006). These findings suggest that, Mycobacterium may have evolved mechanisms that help it to evade T cell responses and to persist in the system.

2.7 BCG mediated modulation of autoimmune diabetes

BCG-mediated suppression of autoimmune diseases like autoimmune diabetes (Harada et al., 1990) and EAE (Ben-Nun et al., 1995; Sewell et al., 2003) in mice, is by virtue of the protein and lipid constituents of Mycobacterial cell

envelope, which are capable of activating host adaptive immunity. BCG-derived protein antigens promptly activate MHC restricted $CD4^+$ and $CD8^+$ T cells, while the BCG-derived lipid-antigen elicits the activation of CD1 restricted T cells, monocytes and macrophages in humans (Watanabe et al., 2006). Mycobacteria-induced switch in the cytokine profiles of islet-infiltrating lymphocytes from a Th1-associated to a Th2-associated pattern appeared to mediate diabetes prevention in NOD mice (Shehadeh et al., 1993). Harada and colleagues reported that a single intravenous injection of 0.25-1 mg ($4x10^7$ CFU) of live BCG at approximately 10 weeks of age produced a potent suppression of insulitis and overt diabetes in NOD female mice, and this protection could be adoptively transferred to naïve recipients by spleen cells (Harada et al., 1990). This transferred protection was associated with plastic adherent cells, which expressed CD11b, but not CD 90 (Yagi et al., 1991). Comparably, a single injection of CFA, in NOD mice less than 12 weeks of age, prevented the onset of diabetes (Qin et al., 1993; Sadelain et al., 1990). They suggested that nylon-wool non-adherent, $CD90^-$, radio resistant leukocytes imparted this protection. The protective ability of splenocytes was lost on depleting these cells from the donor and recipient mice with monoclonal antibody (mAb) specific for asialo GM1, while the adoptive transfer of CD49b ($DX5^+$), $CD3^-$ leukocytes reconstituted it. These findings suggested that NK cells were the likely population of cells that imparted Mycobacteria-induced diabetes-protection in NOD mice (Lee et al., 2004).

IFN-γ was not essential for diabetes development in NOD mice (Serreze et al., 2000). Recombinant IFN-γ (rIFN-γ) administration reduced diabetes incidence and insulitis in 5-week-old NOD mice in a dose dependent manner, with the highest dose (10^5 units i.p for 5 weeks) suppressing the final diabetes incidence to 42% from the 74% incidence of control mice (Sobel et al., 2002). Furthermore, they observed low ConA-induced proliferation of responder cells co-cultured with NOD spleen cells preincubated with rIFN-γ compared with splenocytes preincubated in media without rIFN-γ. They also reported that splenic cells from rIFN-γ treated NOD mice expressed higher levels of intercellular adhesion molecule (ICAM 43% higher), B7-2 (35% higher) and MHC-1 (28% lower) than in control mice. von Herrath and colleagues using transgenic C57BL/6 and BALB/c mice, reported that absence of IFN-γ led to insufficient upregulation of MHC class I molecule on β-cells and class II molecules on APCs, ablating the induction of IDDM (von Herrath and Oldstone, 1997). This was based on their finding that targeted mutant mice deficient in IFN-γ, and transgenically expressing LCMV nucleoprotein (NP; slow - onset IDDM) or glycoprotein (GP; rapid - onset IDDM) in their β-cells, under the influence of rat insulin promoter (RIP GP 34-20 / RIP NP 25-3), did not develop IDDM when compared to IFN-γ-competent RIP GP or NP control mice. Over expression of suppressor of cytokine signaling-1 (SOCS-1) in β-cells completely prevented diabetes in NOD8.3 TCR transgenic mice carrying the rat insulin promoter suppressor of cytokine signaling-1 (RIP- SOCS-1) transgene, but were not protected from accelerated diabetes induced by the adoptive transfer of

splenocytes from diabetic NOD mice indicating that IFN-γ in the host is required for the protection (Chong et al., 2004). These findings suggested that IFN-γ played a suppressive role in autoimmune diabetes. BCG/CFA mediated diabetes protection in animal models was dependent on the production of IFN-γ, as CFA/ *M.bovis* -treated, IFN-γ deficient NOD.*Ifng*$^{-/-}$ mice were not protected from diabetes (Serreze et al., 2001).Qin and co-workers who studied the adoptive transfer of diabetes out of spontaneously diabetic, BCG-treated NOD mice, arrived at a similar conclusion. Three hundred (300μg) BCG administration in diabetic NOD donors reduced 75% disease in NOD.*scid* and irradiated NOD recipients by 75%, and this effect was mostly eliminated by protein-mediated blockade of IFN-γ signalling. Moreover, the injection of 20μg of rIFN-γ over 10 days partially replicated the inhibitory effects of BCG on the efficacy of diabetes transfer (Qin et al., 2004). These findings indicate a primary role for IFN-γ in BCG immunotherapy.

BCG treatment enhanced the expression of FAS, FASL, TNF and its receptor on splenic T cells, while anti-FASL or anti-TNFR1 mAb treatment significantly decreased the apoptosis of BCG-immunised diabetic NOD splenocytes, i*n vitro* (Qin et al., 2004), suggesting that BCG-induced apoptosis of diabetogenic T cells imparted the efficacy of this treatment. These findings were consistent with the results of Novelli and colleagues (Novelli et al., 1996), who hypothesised that BCG inhibits diabetes by stimulating the secretion of IFN-γ, which induces apoptosis of activated T cells in a FAS-dependent mechanism. Similarly, Martins and co-workers (Martins and Aguas, 1999) observed an increase in FAS, FASL and IFN-γ

levels in the splenocytes of NOD mice infected with *M. avium* (10^8 viable bacilli per mouse). They also reported an increase in the number and cytotoxic activity of FASL expressing splenic CD4 and CD8 T cells in immunised NOD mice as opposed to uninfected controls, affirming the role of FASL and IFN-γ in Mycobacteria mediated diabetes protection.

2.7.1 BCG influence immunoregulatory functions of NKT cells

NKT cells are a subset of T cells with a limited TCR repertoire, restricted by a nonclassical MHC class I molecule (Bendelac et al., 1995). In C57BL/6 mice, BCG mediates a shift in the density of IL-4 producing $CD4^+$ NKT cell population of the liver to a low-density population secreting large amounts of IFNγ. Chen and colleagues (Chen et al., 2005) reported that activated NKT cells could inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. $FASL^+$ iNKT cells may act as ready-to-kill sentinels of innate immunity, but at the same time assist in tolerance induction by eliciting FAS/FASL-mediated apoptosis of antigen carrying B cells (Nowak et al., 2006). FAS expression in the liver is upregulated on activated NKT cells following Mycobacterial infection, with the NKT cells becoming more susceptible to apoptosis (Chiba et al., 2008).

2.7.2 BCG mediated reversal of T1D

Experimental evidence in mice suggests that spleen cells could reverse autoimmune diabetes by replacing lost islet cells through trans-differentiation when injected together with an immune adjuvant (CFA) into diabetic NOD mice (Kodama et al., 2003). However, the contribution of splenocytes towards islet regeneration was later ruled out by three other groups (Chong et al., 2006; Nishio et al., 2006; Suri et al., 2006), but each of these three studies supported the conclusion that an adjuvant dependent dampening of autoimmunity coupled with the regeneration of residual host islets led to the reversal of autoimmune diabetes in mice. They observed circumferentially distributed $CD4^+CD25^+FoxP3^+$ regulatory T cells mediated the dampening of autoimmunity in the diabetic mice. Following the success of BCG treatment in mice - subcutaneous injection of BCG into diabetic patients with recent onset of disease was tested. Shehadeh and colleagues (Shehadeh et al., 1994) administered 0.1 mL of BCG (1mg/ml) in 17 newly diagnosed T1D patients, almost all of whom returned to their diabetic state within a year of treatment. However, they reported increases in the frequency and duration of the "honeymoon period", based on which other randomised clinical trials (RCTs) were held. A multicentre RCT in Europe which compared the effect of BCG + nicotinamide therapy with nicotinamide alone controls in 72 newly diagnosed IDDM patients, indicated no difference in the remission rates in the two groups (Pozzilli, 1997). Elliot and colleagues randomised 26 newly diagnosed diabetic New Zealanders to receive BCG or saline. Their data suggested no

evidence of an effect on C-peptide levels or the clinical course of the disease (Elliott et al., 1998). In an RCT conducted in USA by Allen and colleagues (Allen et al., 1999), 94 children with new onset diabetes received BCG or a placebo. The authors reported no difference in C-peptide levels, insulin requirements or HbA1c levels subsequent to treatment. However, one has to bear in mind that doses used to prevent autoimmune diabetes in NOD mice are 10,000 times greater as a proportion of body weight than the dose used in these clinical trials. This suggested that a higher dose (includes possibility of complications) or multiple low doses of BCG could be more effective. The results from a recent RCT of 6 adults with long-term T1D randomly assigned to two low-dose 0.1 ml BCG (1.6–3.2×10^6 CFU/injection) or saline placebo treatment, administered 4 weeks apart, indicated elevated C-peptide levels compared to controls (Faustman et al., 2012). This finding is consistent with the successful intervention in mice and suggested that BCG could have potential value in the treatment of T1D in humans.

2.8 Mouse models of systemic autoimmunity

Many animal models have been generated to study the mechanism of autoimmune mediated tissue destruction leading to organ specific and systemic autoimmune disease. BB rats and NOD mice remain the most widely used and best-characterised animals for modelling the aetiological, immunological, pathological and genetic aspects of autoimmune diabetes (Mordes et al., 2004).

Although NOD mice are commonly used as a model of autoimmune diabetes mellitus, they are also prone to the spontaneous development of a number of other autoimmune phenomena in senescence. These include antinuclear antibodies (ANA) and haemolytic anaemia (Baxter and Mandel, 1991; Humphreys-Beher et al., 1993). While a single dose of heat killed BCG prevents diabetes, it accelerates the precipitation of a form of autoimmunity similar to human systemic lupus erythematosus (SLE). BCG-mediated autoimmunity in NOD mice is characterised by autoimmune haemolytic anaemia (AIHA), raised titres of anti-nuclear autoantibodies (both anti-ds-DNA and anti-Sm autoantibodies) in sera and immune complex deposits in renal glomeruli consistent with glomerulonephritis. *M. bovis* mediated induction of lupus in NOD mice is being used as a model for studying the etiopathogenesis of this disease. Some of the murine/rat models that develop systemic autoimmunity similar to that of lupus in NOD mice are briefly described in this section.

2.8.1 *lpr* and *gld* mice

The lymphoproliferation-associated mutations *lpr* and *gld* (generalised lymphoproliferation) spontaneously occurred in inbred lines of mice and are recessive, loss of function mutations (Andrews et al., 1978; Roths et al., 1984) of the *Fas* and *Fasl* genes respectively (Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). The lymphoproliferative disorder in MRL$^{lpr/lpr}$ and C3H$^{gld/gld}$ mice is characterised by the accumulation of an unusual population of DN ($CD4^-CD8^-$),

$B220^+$, $J11d^+$, heat stable antigen (HSA^+) T cells in the periphery, over-production of IgG and IgM antibodies, lymphadenopathy and splenomegaly (Cohen and Eisenberg, 1991). *Lpr*/*gld* syndrome shares many characteristics of the human disease systemic lupus erythematosus (SLE). Mice and humans carrying mutations in *FasL* that prevent FAS ligation exhibit disrupted lymphocyte homeostasis, resulting in massive lymphoaccumulation and a systemic autoimmune syndrome similar to lupus.

Unlike other *lpr/gld* mice strains, which only develop lymphadenopathy and splenomegaly, MRL mice manifest nephritis or arthritis and die at around 5 months of age (Andrews et al., 1978; Izui et al., 1984). Allen and colleagues observed that although *lpr* and *gld* mutations are non-allelic, they exhibit a similar phenotype. In a series of *in vivo* experiments involving bone marrow chimaeras of *lpr, gld* and wild-type mice, they demonstrated that *lpr* and *gld* represented the genes for a ligand-receptor pair (Allen et al., 1990). This model also suggested the *lpr* product to be a receptor expressed in both bone marrow (BM) and peripheral cells, and considered *gld* product to be a soluble cytokine or membrane associated protein expressed on the BM derived cells. Watanabe and colleagues mapped the CD95 gene to a location near the *lpr* locus on the mouse chromosome 19 (Watanabe et al., 1991). Southern blot hybridisation and restriction mapping performed by Adachi and colleagues revealed that an approximately 5.4-kb DNA fragment was inserted 3.5 kb upstream of exon 3 in the FAS gene in *lpr* mice. On

sequencing, the inserted element was found to be homologous to a mouse early transposon (ETn) - RMg2 ETn, indicating that an RMg2-like ETn has been inserted in intron 2 of the CD95 gene of *lpr* mice (Adachi et al., 1993). Reverse transcription polymerase chain reaction at particular position of FAS-mRNA consistently yielded a longer product (around 180 base pair longer) in *lpr* thymocytes, when compared to the wild type mice, confirming insertions in the FAS gene intron (Kobayashi et al., 1993).

Northern hybridisation analysis indicated lower FAS-mRNA level in the liver and thymus of *lpr* mice when compared to the wild type (Watanabe-Fukunaga et al., 1992). Similar analysis by Wu and colleagues indicated higher expression of ETn RNA in the thymus of MRL$^{lpr/lpr}$ than MRL$^{+/+}$ mice. They generated a CD2-FAS transgenic mouse model in which murine FAS cDNA was expressed under the regulation of the CD2 promoter and enhancer to correct defective FAS expression in T cells of MRL$^{lpr/lpr}$ mice (Wu et al., 1993). Elimination of glomerulonephritis and development of lymphadenopathy, decrease in the levels of autoantibodies and reduced thymic ETn expression was observed in this model (Wu et al., 1993; Wu et al., 1994), suggesting that early correction of the abnormal FAS expression could retard the acceleration of autoimmune disease. The reduction in FAS gene expression was attributed to the premature termination and aberrant splicing of the FAS gene transcript caused by the polyadenylation signal (AATAAA) carried on the long terminal repeat of the ETn. Adachi and colleagues noticed a considerable

reduction in expression efficiency, when this ETn was inserted into the corresponding intron in a mammalian expression vector (Adachi et al., 1993). The *lpr*^{cg} gene is allelic to *lpr*, with a T-A point mutation in the FAS cytoplasmic region, causing an isoleucine to be replaced by an asparagine. Though *lpr*^{cg} mice express normal size FAS mRNA at levels similar to that of wild type mice (Watanabe-Fukunaga et al., 1992), its ability to transduce the apoptotic signal remains impaired, indicating that *lpr* mutations are loss of function mutations of the FAS gene. The *gld* mutation arose in the inbred C3H/HeJ strain of mice at the Jackson Laboratories (Roths et al., 1984). Watson and colleagues mapped the *gld* mutation to a less than 1 cM segment on distal mouse chromosome 1 (Watson et al., 1992), near which the CD95L gene was localised (Takahashi et al., 1994). *FasL* of *gld* mice carries a point mutation in the C-terminus of the coding region, causing a phenylalanine to be replaced by leucine in the extracellular region, thereby abrogating its ability to bind to FAS. Recombinant *gld* CD95L expressed in COS cells could not induce apoptosis in cells expressing CD95, indicating that *gld* is a loss of function mutation (Takahashi et al., 1994).

2.8.2 Marshall-Clarke and Playfair model of murine AIHA

Autoimmune haemolytic anaemia (AIHA) was one of the first diseases that was shown to have an autoimmune pathology, and has been described in a number of species, including humans, dogs, cats, rabbits and cattle (Loutit and Mollison, 1946). AIHA is an acquired immunologic disease in which premature haemolysis

of autoantibody sensitised erythrocytes occur via the complement and the reticuloendothelial system (RES). AIHA in humans is often characterised by an insidious onset (particularly when extravascular haemolysis predominates) followed by a waxing and waning course (Dacie, 1962; Gehrs and Friedberg, 2002; Pirofsky, 1976). It may occur as a primary (idiopathic) disorder or coexist with some other underlying disease process like lymphoproliferative disorders, systemic lupus erythematosus (SLE), infectious mononucleosis, viral pneumonia or cytomegalovirus infection. The common symptoms of AIHA include weakness, pallor, fatigue, jaundice and frequent occurrence of mild splenomegaly (Mack and Freedman, 2000).

There exist examples of AIHA in laboratory mice (Helyer and Howie, 1963; Playfair and Marshall-Clarke, 1973), which either could be spontaneous or induced models of the disease. The New Zealand Black (NZB) mouse develops AIHA spontaneously (Barker et al., 1993a; Helyer and Howie, 1963). The disease can also be induced in other healthy murine strains by repeated immunisation with rat RBCs (Barker et al., 1993b; Cox and Keast, 1974; Naysmith et al., 1980; Playfair and Marshall-Clarke, 1973), or by infection of C3HeB/FeJ mice with the docile strain of lymphocytic choriomeningitis virus (LCMV). In all the above models, the condition is a classic example of type II hypersensitivity, characterised by autoantibodies that bind to RBC and destroy them via phagocytosis by splenic macrophages and/or complement-mediated lysis. The warm AIHA caused by IgG antibodies in

these experimental animals provides us with a well-characterised model to study the loss of immunological self-tolerance in this form of autoimmunity.

The experimental model of murine AIHA was developed from the original observation of Playfair and Marshall-Clarke (1973) that mice injected with RBC from rats produce RBC auto Abs, which was later confirmed by several other groups (Cox and Keast, 1974; Naysmith et al., 1980). They observed that after three intravenous or intraperitoneal injections of $2x10^8$ rat RBCs at weekly intervals, mice of many strains were positive for Coombs' Abs. The disease developed was reported to be similar to AIHA in man, with evidence of anaemia, reticulocytosis, shortened erythrocyte survival and a high incidence of autoantibody production. T cell bypass model proposed by Allison, Weigle and their colleagues (Allison et al., 1971; Weigle, 1971)could explain the mechanism of induction of RBC autoantibodies in the experimental model under discussion. It was postulated that the untreated mice harbours B-lymphocytes, which recognise RBC autoantigens that are homologous to the antigenic determinants on rat RBC. However, these B cells remain unreactive owing to a lack of signalling from autoreactive T cells that have been eliminated or suppressed. On immunisation, the homologous antigenic determinants on rat RBCs are presented to the host with non-self-structures recognised by other T cells, thereby bypassing the absence of functional self-reactive T cells. These results support the idea that human AIHA may be induced by exogenous antigen stimulation (Dacie and Worlledge, 1969).

2.8.3 Anti-RBC autoantibody transgenic mice

Okamoto and colleagues (Okamoto et al., 1992)generated transgenic mice expressing the pathogenic IgM anti-mouse RBC (MRBC) autoantibody 4C8. Most mice in this model demonstrated a dramatic reduction in the number of B cells expressing the anti MRBC transgene by anergy or deletion in spleen and lymph nodes. Despite this marked diminution of the number of B cells nearly 50% of the mice were reported to develop AIHA. In these mice anti-MRBC autoantibodies were solely generated by $CD5^+$ B cells (or B1 cells) present in the peritoneal cavity, where the MRBC autoantigens were absent. Murakami and co-workers (Murakami et al., 1992) reported a reduction of AIHA, following the selective elimination of $CD5^+$ B cells by injection of MRBC into the peritoneal cavity, in these mice. They also observed apoptosis of the transgene expressing $CD5^+$B cells after the intraperitoneal injections. These findings suggested that sequestration of anti-MRBC autoreactive $CD5^+$B cells behind an anatomical barrier underlies the development of AIHA in this transgenic model. To confirm the role of B-1 cells in induction of AIHA male F1 mice were generated by crossing antiRBC autoAb-Tg with CBA/N which bear the X-linked immunodeficiency mutation (*xid*). These Tg mice which lack the B-1 cells due to the *xid* mutation did not suffer from AIHA (Murakami et al. 1994), suggesting the direct involvement of B-1 cells in the pathogenesis of AIHA in this model. Reduced occurrence of autoimmune symptoms was reported in NZB/*xid* and NZB/WF1/*xid* which congenitally lacked B1 cells due to *xid* gene (Steinberg et al., 1982). Elimination of B1 cells by repeated

injection of anti-IL-10 Ab *in vivo* prevented and ameliorated the autoimmune symptoms in NZB/WF1 mice (Ishida et al., 1994). Murakami and colleagues selectively depleted peritoneal B1 cells by hypotonic shock with repeated intraperitoneal injection of distilled water once every week. In treated NZB mice autoAb production was decreased and the occurrence of AIHA was prevented (Murakami et al., 1992), thus suggesting that peritoneal B1 cells may be the source of auto Ab production in AIHA of NZB and NZB/WF1 mice. Environmental factors may be involved in the triggering of autoreactive B cells, and this could account for the fact that only half of the transgenic mice developed AIHA. The frequency of AIHA in anti RBC autoAb Tg mice varied depending on the housing conditions. 50% of mice housed in conventional conditions suffered from AIHA while none of the ones housed in a specific pathogen free condition developed the disease (Murakami et al., 1997). Injection of bacterial lipopolysaccharides induced AIHA in asymptomatic transgenic animals. Oral administration was reported to be the most effective mode when compared to intraperitoneal or intramuscular administration, in activating peritoneal and lamina propria B1 cells (Murakami et al., 1994), thereby suggesting that bacterial infection may be responsible for triggering the AI disease that occurred spontaneously in half of the Tg mice housed under conventional conditions.

2.8.4 New Zealand Black (NZB)

NZB strain was one of the first experimental animal models of autoimmunity. Mice from this strain are genetically predisposed to developing haemolytic anaemia, associated with anti-erythrocyte auto Abs (AEA), decrease in haematocrit values, reticulocytosis, hemoglobinemia and splenomegaly, thus resembling the human counterpart of the disease (Bielschowsky et al., 1959; Helyer and Howie, 1963). Holmes and colleagues (Holmes et al., 1961) had reported that this condition could as well be transferred to young isologous mice by inoculating splenocytes from older mice with signs of the disease. Based on genetic studies on the crosses of NZB and non-autoimmune New Zealand Chocolate mice it was reported that 100% of the F1 and 74% of the F2 hybrids developed AIHA (Bielschowsky and Bielschowsky, 1964), suggesting the presence of a single dominant gene controlling AEA in NZB . Knight and Adams (Knight and Adams, 1981) hypothesised that a single dominant susceptibility allele Aia-1 (autoimmune anaemia locus) on chromosome 4 in NZB strain contributed to the development of AEA. In most mouse strains, except for NZB and NZC the effect of this gene is suppressed to a varying degree by wild type modifying dominant alleles. Ochiai and colleagues (Ochiai et al., 2000) using microsatellite markers in the (C57/BL6 x NZB) F1 x NZB backcross progeny, mapped C57/BL6 modifying loci for AEA. It was observed that generation of AEA was down regulated by a combined effect of two major independently segregating dominant alleles, one (Aem-2) linked to D7MIT30 on Chromosome 7 and the other (Aem-3) linked to

D10MIT42 on chromosome 10, while splenomegaly was modified mainly by a C57/BL6 allele (Spm1) linked to *D4MIT5*8 on chromosome 4. These findings suggested a multigenic control of AIHA in NZB mice. Autoimmune response to erythrocytic antigens in NZB mice have been reported to be heterogeneous. Two major groups of autoantigens initially discovered were the X antigens and the HB antigens. Barker and colleagues (Barker et al., 1993b) reported self-reactive antibodies against the erythrocyte Band 3, an anion transporter found in the erythrocyte membranes, to be responsible for spontaneous AIHA in NZB mice. They studied this Ab this using eluates from Coombs-positive erythrocytes to immunoprecipitate a molecule that was detected with Abs against the band 3 protein.

2.8.5 Other murine models of AIHA

Viral infections are involved in the pathogenesis of blood related AI diseases such as AIHA in experimental models (von Herrath and Oldstone, 1996). The docile strain of LCMV triggers a peripheral haemolytic anaemia in C3HeB/FeJ mice that is correlated with the production of anti-erythrocyte antibodies recognising the band-3 antigen (Mazza et al., 1997). Lactate dehydrogenase-elevating viral (LDV) infection in mice immunised with rat erythrocytes resulted in faster anti-RBC autoantibody response, without enhancement of anaemia (Verdonck et al., 1994). Haemolytic anaemia can be induced into normal mice by administration of monoclonal antierythrocyte autoantibodies like 34-3C and 4C8 IgG_{2a}, which induce

it through phagocytosis of opsonised erythrocytes by cells of the reticule-endothelial system and 31-9D IgG, which trigger agglutination of RBCs sequestered in the spleen and liver (Fossati-Jimack et al., 1999).

2.8.6 Modulation of autoimmune hemolytic anaemia by IFN-γ

Berney and colleagues (Berney et al., 1992) reported an enhancement of AIHA mediated by antierythrocyte monoclonal Ab due to macrophage stimulation by cytokines or growth factors. Viral infections frequently trigger the secretion of IFN-γ (Markine–Goriaynoff et al., 2000), a potent macrophage activator. IFN-γ is also reported to be involved in the exacerbation of autoantibody mediated HA or thrombocytopenia induced by LDV, as an absence of disease was observed in mice deficient for its receptor (Musaji et al., 2004). This evidence suggests that microbes may trigger autoantibody-mediated anaemia by activating macrophages through IFN-γ production, a mechanism that could also account for the pathogenic similarities of multiple infectious agents. T helper cells from AIHA patients and NZB mice proliferating *in vitro* against the respective RBC autoantigens or peptides, secrete predominantly IFN-γ, with little or no IL-4 (Hall et al., 2002; Shen et al., 1996). The amelioration of disease observed in NZB mice on nasal administration of soluble peptide containing the dominant helper epitope, was associated with a deviation of response from an IFN-γ to IL4 producing T cell population (Shen et al., 2003), suggesting a pathogenic role for IFN-γ in autoimmune haemolytic anaemia.

2.9 Summary and Rationale

T1D is a well-characterised autoimmune disease, and recent efforts to modulate the adaptive immune system by antigen-based immunotherapies have succeeded in protecting animal models against developing the disease (Peakman and von Herrath, 2010). However, the stochastic nature of pathogenic and tolerogenic antigen selection in mouse and humans could prevent the successful translation of these protocols to patients at the time of diagnosis. Recent evidence suggests components of the innate immune system to be involved in the precipitation of autoimmune disease, including T1D. Considering its crucial role in orchestrating adaptive immune responses, it is probable that the innate arm of the immune system could influence autoimmunity. Hence, innate immune cells or components of its signalling pathways should be potential targets, therapeutic manipulation of which could inhibit the autoimmune processes leading to T1D. TNF superfamily of receptors and their ligands play crucial roles in immune surveillance and tolerance, and hence are critical in regulating autoimmunity. TRAIL, a key ligand of the TNF superfamily is implicated in the abrogation of autoimmune disease like T1D, autoimmune arthritis and EAE. However, on the contrary, TRAIL exacerbated parent into F1 graft-versus-host disease, and blockade of this ligand within the CNS suppressed EAE, suggesting a pleotropic nature of this molecule. FAS ligand, apoptosis inducing ligand of TNF superfamily play an important role in the regulation of the immune processes, especially T-cell homeostasis. Spontaneous gene mutation of FAS (*lpr* mutation) or FASL (*gld*

mutation) that rendered these molecules non-functional, inhibited autoimmune diabetes in NOD mice. However, there are reports that suggest enhanced FAS-FASL signalling to be beneficial in suppressing diabetogenic cells. Thus, the contradictory reports on the roles of TRAIL and FASL in the pathogenesis of autoimmunity raise doubts on their effects in T1D. Since TNF superfamily ligands are suppressors of innate immunity, a major contributor of T1D pathogenesis, there exists a possibility that islet-TRAIL/FASL expression could exert unknown beneficial effects. Moreover, no clear evidence suggests a direct role for these apoptosis-inducing ligands in the process of β-cell destruction. In this study, TRAIL deficient NOD mice, and NOD mouse lines with allelic *Fasl* expression will be used to identify and characterise TRAIL and/or FASL-associated mechanism(s) that could control islet susceptibility to immune destruction in T1D. IFN-γ, though not detrimental to β-cells by itself, contributes towards β-cell apoptosis in association with IL-1β and TNF. This cytokine has also been implicated in the precipitation of other forms of systemic autoimmunity. Interestingly, mycobacteria-induced secretion of IFN-γ appeared to protect NOD mice from diabetes, the mechanism(s) of which is still not clearly explained. NOD mice with targeted deletional mutation for IFN-γ and its receptor β chain will be used in order to investigate the role of this cytokine in autoimmune diabetes modulation and in the precipitation of other forms of systemic autoimmunity.

.....

CHAPTER 3

.....

MATERIALS AND METHODS

3.1 Sources and housing of mice

Breeder pairs of NOD/ShiLt (stock number 001289; abbreviated NOD/Lt), NOD.CB17-*Prkdc*^{scid}/J (stock number 001303; abbreviated NOD.*Scid*) mice and targeted gene-deficient mutants of *Ifng* (NOD.*Ifng*$^{-/-}$) and *Ifngr2* (NOD.*Ifngr2*$^{-/-}$) were originally obtained from the Jackson Laboratories. NOD mouse lines bearing targeted mutations for TLR 2 (NOD.*TLR2*$^{-/-}$) was generated by Shahead Chowdhury by backcrossing from Shizo Akira's C57BL/6 stocks to NOD/Lt mice.

Other mice strains used in the studies were NOD.*Nkrp1*^{b}, carrying B6-derived alleles at the NK complex on chromosome 6 (from *D6mit105* to *D6mit135*), permitting the use of the NK1.1 marker. Mice carrying mutant alleles of *D1Bax208*, spontaneous mutation identified in a tandem repeat sequence adjacent to *Fasl,* in inbred NOD line (originally purchased from Jackson laboratory Maine, USA and from Animal Resource Centre (ARC) in Perth), named as NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2}. Targeted gene-deficient mutants of *Tnfsf10* (tumor necrosis factor (ligand) superfamily, member 10), backcrossed from C57BL/6 mice onto the NOD/ShiLt background (NOD/ShiLt.*Tnfsf10*$^{-/-}$ (abbreviated NOD.*Tnfsf10*$^{-/-}$)), in association with our collaborators Mark Smyth (Peter MacCallum cancer centre, Melbourne) and Thomas Kay (St. Vincent's institute of medical research, Melbourne). All the above mice were housed in specific pathogen free conditions, in rooms with HEPA-filtered air maintained at 19 ± 1.5^{0}C at the Immunogenetic Research Facility of James Cook University. Sentinel mice were tested at 4-month intervals for a panel of viral, bacterial and mycoplasmal pathogens including

mouse hepatitis virus, rotavirus, ectromelia virus, mouse cytomegalovirus, polyoma virus, murine adenovirus, lymphocytic choriomeningitis virus, mouse pneumonia virus, reovirus, Sendai virus, Theiler's murine encephalitis virus, *Bacillus piliformis*, *Mycoplasma pulmonis*, *Bordtella bronchiseptica*, *Corynebacterium kutscheri, klebsiella* species, *Pasturella multocide*, *Pasturella pneumotropica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Citrobacter freundii,* and *salmonella* species. No mice tested positive for any of these pathogens.

3.2 Cellular Biology

3.2.1 Organ removal

Mice were sacrificed by CO_2 asphyxiation or cervical dislocation prior to organ harvest. Organs were collected into MACS buffer (PBS containing 2mM EDTA (Amresco) and 0.5% (w/v) BSA (ICN Biomedicals), adjusted to a pH of 7.4) and kept in ice during preparation. Approval for all animal experimentation was obtained from the Institutional Ethics Committee at the James Cook University.

3.2.2 Adoptive Transfer of diabetogenic splenocytes

Diabetogenic splenocytes were obtained from newly diagnosed diabetic male and/or female NOD/Lt, NOD.*Tnfsf10*$^{-/-}$, NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2}, NOD.*Ifng*$^{-/-}$, NOD.*Ifngr2*$^{-/-}$, and NOD.*Nkrp1*^{b} mice respectively. After removing the

erythrocytes by treatment with RBC lysing buffer (Sigma-Aldrich), cells were counted using a Z1 Coulter particle counter (Beckman Coulter). The diabetogenic cells were resuspended in saline and 15-20x10^6 cells in 200ml PBS were injected intravenously into 8-10 week old NOD.*scid* females or into preirradiated (7Gy) male and/or female NOD.*Tnfsf10*$^{-/-}$, NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2} and NOD/Lt recipients. In case of titration experiments, preirradiated male and female recipients from either group were injected with various doses (20x10^6 cells, 16x10^6 cells, 12x10^6 cells, 8x10^6 cells and 5x10^6 cells) of diabetogenic splenocytes.

3.2.3 Cell suspension preparation for flowcytometry

Cell suspensions from thymus were prepared by gently grinding the thymus between frosted microscope slides. Thymocytes were released into ice chilled MACS buffer (PBS containing 2mM EDTA (Amresco) and 0.5% (w/v) BSA (ICN Biomedicals), adjusted to a pH of 7.4). Splenocytes were prepared by disrupting the spleen using a 26-gauge needle and forceps in MACS buffer, and the resulting cell suspension was treated with RBC lysing buffer. Lymphocytes were released from the lymph nodes by carefully tearing open the capsule using two 26-gauge needles and then gently grinding the capsule between two frosted microscope slides in ice-cold MACS buffer. Finely cut liver was ground on a sieve using the plunger of a 5ml syringe and filtered through with cold saline. A 33.75% percoll (GE Healthcare) gradient was used to isolate hepatic lymphocytes from the liver cell suspension. A RBC lysis step was carried out when necessary.

3.2.4 Surface staining of leukocyte subsets for flowcytometry

Cells were labelled with the following antibodies for flowcytometric analyses:

Antibody	Conjugate	Specificity	Clone	Company
anti-βTCR	APC	Mouse	H57–597	BD Pharmingen
anti-CD4	Pacific Blue	Mouse	RM4-5	BD Pharmingen
anti-CD25	Biotin	Mouse	7D4	BD Pharmingen
anti CD3	APC	Mouse	145-2C11	BD Pharmingen
anti CD8	PerCP	Mouse	53-6.7	BD Pharmingen
anti CD45	PerCp-Cy5.5	Mouse	3F11	BD Pharmingen
anti CD44	FITC	Mouse	IM7	BD Pharmingen
anti CD3	PE	Mouse	500A2	BD Pharmingen
anti CD4	APC-Cy7	Mouse	GK1.5	BD Pharmingen
anti CD8	APC-Cy7	Mouse	53-6.7	BD Pharmingen
anti CD62L	APC	Mouse	MEL-14	BD Pharmingen
anti CD49b	Biotin	Mouse	DX5	BD Pharmingen
anti Vβ5.1,5.2 TCR	PE	Mouse	MR9-4	BD Pharmingen
anti Vβ3 TCR	PE	Mouse	KJ25	BD Pharmingen
anti-IL-17	PE	Mouse	TC11-18H10	BD Pharmingen
anti CD3	PE-Cy7	Mouse	145-2C11	BD Pharmingen
anti NK1.1	FITC	Mouse	PK136	BD Pharmingen
Anti TNF	FITC	Mouse	MP6-XT22	BD Pharmingen
anti-TRAIL (CD253)	PE	Mouse	N2B2	Biolegend
anti-FASL	FITC	Mouse	FLIM 58	MBL, Japan
Anti-FOXP3	FITC	Mouse	FJK-16s	eBioscience
Anti NK1.1	FITC	Mouse	PK136	BD Pharmingen

Table 3.1 List of antibodies used in staining mouse cells for flowcytometric analyses.

Mouse CD1d tetramer conjugated to PE and loaded with α-GalCer was produced at Dale Godfrey laboratory based on Matsuda and colleagues (Matsuda et al., 2000). For surface staining, antibodies were diluted in FACS buffer. Cells were preincubated for 20 min with CD16/32 (Fc Block) to prevent FcR binding, before addition of surface staining Ab cocktails. Viable lymphocytes were identified by the forward and side scatter profile and in some cases by propidium iodide (PI) exclusion. A forward scatter-area against forward scatter-linear gate was used to exclude doublets from analysis. Flow cytometry was performed on a CyAn ADP flowcytometer (DakoCytomation) or FACSCalibur (BD Bioscience) and analysed with Summit 4.3 software (DakoCytomation) or Kaluza 1.2 analysis software (Beckman Coulter, CA).

3.2.5 Intracellular staining of leukocyte subsets for flowcytometry

In the case of intracellular staining, the surface molecules were initially stained as described above. The cells were then fixed/permeabilised for 3 hours using the fixation/permeabilisation buffers (Biolegend CA) for FoxP3 staining after which they were washed and incubated for 15 min with CD16/32 (clone 93; eBioscience). This was followed by incubation with the anti-mouse Foxp3Ab in permeabilisation buffer. For IL-17 and TNF staining, splenocytes were initially incubated with PMA (500ng/ul) and ionomycin (500ng/ul) for 6 hours in the presence of BD GolgiPlug. The surface molecules were then stained as described earlier. The cells were then fixed/permeabilised in BD Cytofix/Cytoperm™

solution for 10 – 20 min at 4°C. This was followed by incubation with the anti-mouse IL-17 and anti-mouse TNF antibodies in FACS buffer.

3.2.6 NK cell depletion

NK cells from diabetogenic splenocytes were depleted by magnetic cell sorting using the CD49b MicroBeads or anti FITC MicroBeads (Miltenyi Biotec GmbH). Splenocytes incubated with CD49b MicroBeads were passed through MS columns (Miltenyi Biotec GmbH) placed in the magnetic field of a MiniMACS separator (Miltenyi Biotec GmbH). Unlabelled cells (devoid of NK cells) that passed through the column were collected and purity determined by flowcytometry. Alternatively, splenocytes from NOD.*Nkrp1*^{b} mice were initially incubated with anti NK1.1 FITC antibody, and then with anti-FITC MicroBeads. FITC labelled cells were depleted by passing through MS columns placed in the magnetic field of a MiniMACS separator. Unlabelled cells (devoid of NK cells) that passed through the column were collected and purity determined by flowcytometry.

3.2.7 Islet isolation

Islets were isolated from 4-6 week old male or female NOD/Lt, NOD.*Tnfsf10*$^{-/-}$, NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. In order to isolate pancreatic islets, mice were killed by CO2 asphyxiation and their peritoneal cavities opened and the pancreata exposed. The common bile duct of each mouse were clamped at its bifurcation immediately proximal to the liver and at the duodenum, and then

infused with 3ml chilled Collagenase P solution (1.5u/ml in Complete HBSS). The pancreata were then excised and digestion started by warming to 37^0C and halted after 16.5 minutes by rapid chilling. The tissues were then disrupted by vigorous shaking for one minute, and the digest filtered (500 um stainless steel mesh), washed in serum free RPMI and centrifuged over Histopaque 1077. Healthy islets were then handpicked into warm CMRL supplemented with 10% foetal calf serum.

3.2.7.1 Surface staining of Islets for flowcytometry

For flow cytometric analysis, islets were dispersed into single cells with 0.25% trypsin (Calbiochem, La Jolla, CA), 10mM EDTA in HBSS, and allowed to recover in culture medium for 1 hour before staining with mAb. Islet cell suspensions were stained with rat anti-mouse TRAIL conjugated to PE (N2B2; Biolegend, CA). Cells were finally washed and resuspended in balanced salt solution with 2% FCS. Analysis was performed on a CyAn ADP flowcytometer (DakoCytomation). Surface TRAIL expression on islets were detected by gating out leukocytes using anti-CD45 (anti-leukocyte common antigen) conjugated to PerCp-Cy5.5 (3F11; BD Pharmingen).

3.3 Molecular Biology

3.3.1 DNA preparation

Extraction of genomic DNA from NOD/Lt, C57BL/6, NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2}mouse strains were carried out using the CAS-1810 X-TractorGene (Corbett Robotics) and the XTR2 X-tractor gene solid sample reagent pack (Sigma, Sydney, Australia). Briefly, DNA was extracted by digesting 11mm tail in 400ul digest buffer (100mM Tris HCl pH 8.0, 10mM EDTA, 100mM NaCl, 0.5% SDS, 50mM DTT, 100mM Proteinase-K), O/N, 56^{0}C, 40 RPM in a VORTEMP 56EVC (Labnet, Hialeah, FL). Samples were lysed by the addition of 700µl 5.25M Guanidine thiocyanate lysis buffer (5.25M Guanidine thiocyanate, 10mM Tris HCl pH 6.5, 20mM EDTA, 4% Triton-X, 64.8mM DTT0, loaded on a glass filter (GF/B) polypropylene microplate (Whatman International, Clifton, USA) and washed 2x in Propanol Wash Buffer and 1x in 100% Ethanol. Samples were eluted in 150 µl Elution Buffer. The DNA yield was quantified spectrophotometrically using NanoDrop ND1000 spectrophotometer (BioRad Laboratories, CA, USA).

3.3.2 Genotyping NOD mice carrying polymorphisms in *D1Bax208*

Identification of simple sequence length polymorphism (SSLP) in NOD/Lt, C57BL/6, NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mouse strains was carried out by genotyping the extracted tail DNA using the following primer pairs: D1Bax208-Forward 5' AGC GAA TGG ATC CAT GAG TG 3' (20bp between 52275-52294 of

gb#AC015932, downstream of *Fasl*, Exon 4), Tm:52°C and D1Bax208-Reverse 5' CAA CCA ACG TTG CTT AGA TCC 3' (21bp between 52445-52425 of gb#AC015932, downstream of *Fasl*, Exon 4), Tm:52°C. Analysis of SSLP polymorphisms were performed on Omn-E thermal cyclers (Hybaid, Basingstoke, UK) followed by ^{32}P end-labelling of the forward primer using 0.3U T4 PNK enzyme (Perkin Elmer). The 10ul PCR mix, included 0.38mM each of dATP, dCTP, dGTP and dTTP(Astral), 1ul 10 x PCR buffer (Roche, Mannheim, Germany), 6mM MgCl2 (Roche), 0.3mM each primer and 0.02U Taq Polymerase(Roche). Products were resolved using polyacrylamide gel electrophoresis, transferred to 3M blotting paper and exposed to Fuji Medical X-ray film O/N.

3.3.3 High Resolution Melt (HRM) analysis

High resolution melt analysis was used to genotype the rederived NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mouse lines. DNA samples were labelled with SYBRGreen™/EvaGreen™, amplified, and HRM analysis performed on a Rotor-Gene 6000 series real time analyser. Melt curves of test DNA sample were compared with that of known genotypes (control DNA samples of NOD/Lt, NOD.*D1Bax208*^{m1} or NOD.*D1Bax208*^{m2}). All samples with similar melting curves (confidence threshold > 85%) were automatically clustered and assigned the respective genotypes (NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2} or WT NOD) by the Rotor-Gene 6000 Series Software v1.7.75 by Corbett Research, a division of Corbett Life Science.

3.3.4 Sequence analysis

Alignment of DNA sequences from *D1Bax208* loci of NOD/Lt, NOD.*D1Bax208*^{m1}, and NOD.*D1Bax208*^{m2} mice was performed by gap-placements using Sequencher (v 4.1, Gene Codes Corporation, Ann Arbor, USA). Transcription Element Search System (TESS), a web tool for predicting transcription factor binding sites in DNA sequences (http://www.cbil.upenn.edu/cgi-bin/tess/tess), was used to predict GATA transcription factor binding sites at the *D1Bax208* loci of NOD/Lt, NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice.

3.3.5 Gene expression analysis

For profiling the gene expression of mouse islets, whole islets from male preinsulitic (3-4 week old) NOD.*D1Bax208m1* and NOD.*D1Bax208m2* mouse strains were isolated by our collaborator Stacey Walters (Shane T Grey's laboratory at the Department of Gene Therapy and Autoimmunity, Garvan Institute of Medical Research, NSW). Samples were stored in RNA-later at 80 C until RNA extraction. My collaborator, Dr Margaret Jordan extracted RNA from these islets for microarray analysis by individually homogenizing them in RLT buffer (Qiagen RNeasy kit). Extensive washing with RNAse-free-DNase-free-water between homogenates minimized any chance of contamination. Samples were passed through Qiashedder columns (Qiagen) to ensure complete homogenization of the tissues and RNA was then extracted using the standard RNeasy protocol (Qiagen). The RNA yields were quantified spectrophotometrically on the

Nanodrop ND-1000 using the convention that 1 absorbance unit at 260nm equals 40ug/ml RNA. Absorbance of samples at 260 and 280 nm was checked and aliquots electrophoresed for determination of sample concentration and purity. A comparison of at least 7 independent islet RNA samples were undertaken to study the gene effects of mice with allelic *Fasl* expression. Dr Margaret Jordan labelled and hybridized 100ng RNA from each sample to Affymetrix Mouse Gene 1.0 ST arrays, that contain more than 750 000 unique probes (representing >28 000 well-substantiated mouse genes). Washing and staining of probe arrays was carried out using the Fluidics Station 450 and the probe arrays were scanned using the Affymetrix 7G scanner. The images (.dat files) were processed using Affymetrix GeneChip Operating Software version 1.4 (GCOS, Affymetrix) and the .CEL files were imported into Partek Genomics Suite 6.0 (Partek Ltd, U.S.A)) using an RMA summarisation algorithm for further analysis. A statistical significance threshold was set by a Mann Whitney U-test and a U-statistic of 0 (i.e. no overlap in signals between the two groups) so as to refine the gene list of highly differentially expressed genes.

3.4 Immunisation / *in vivo* treatment of mice

3.4.1 BCG immunisation

ImmuCyst (Bacillus Calmette-Guérin/Connaught, a freeze dried preparation of an attenuated strain of *Mycobacterium bovis*, Aventis Pasteur, Ontario, Canada), resuspended in phosphate buffered saline (PBS) at a final concentration of 0.1mg, 0.25mg, 0.5mg or 1.0mg per 200µl, was pasteurized at 65^0 C for 40 min and sonicated at 50% of maximum power for 15s using a Branson sonifier 450 (Branson Ultrasonics, Danbury, CT). The homogenized suspension of mycobacteria in saline was injected via the lateral tail vein in the experimental mice at 8 weeks of age, while the control mice received only saline. Any further mention of BCG usage hereafter in this document is assumed to mean ImmuCyst, unless otherwise specified.

3.4.2 Cyclophosphamide treatment

Pre-diabetic male and female NOD.*Tnfsf10*$^{-/-}$, NOD.*Tnfsf10*$^{+/-}$ and NOD.*Tnfsf10*$^{+/+}$ mice intraperitoneally (IP) with Cyclophosphamide (CY, 200mg/kg, Sigma) twice, on day 0 and day 10. This work was carried out by our collaborators Erika Cretney and Anabel Silva, staff at the Peter MacCallum institute, Melbourne.

3.4.3 Streptozotocin treatment

Streptozotocin (SZ, Sigma) was prepared by dissolving in sterile PBS minutes before administration. Pre-diabetic male NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice were challenged IP with a single dose of 120 mg/kg SZ.

3.4.4 α-Galactosylceramide treatment

AlphaGalactosylceramide (αGalCer; Alexis Biochemicals) was prepared in saline supplemented with 0.5% Tween-20 (wt/vol). Eight week old NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2}, NOD.*Tnfsf10*$^{-/-}$, NOD/Lt and NOD.*Cd1d*$^{-/-}$ mice were injected i.v. with 2μg of αGalCer, while the control groups received the vehicle alone (0.5% Tween-20 in saline). The mice were bled by retro-orbital venepuncture 2 h post injection; the whole blood plasma was collected, snap frozen and stored at -80^{0}C for cytokine estimation.

3.4.5 Concanavalin A treatment

Concanavalin A (Con A, Sigma) was prepared by dissolving in sterile PBS as described by Seino and colleagues (Seino et al., 1997), minutes before administration. Pre-diabetic female NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice were injected intravenously with a single dose of 15mg/kg Con A. Hepatic lymphocytes from individual mice were isolated 2 hours post treatment. Flowcytometric analysis of anti-FASL mAb (FLIM58)-stained hepatic T cells and NKT cells were performed to identify levels of FASL expression in these mice.

3.5 *in vitro* assays

3.5.1 Islet-cell-culture

Islets were isolated from 6 week old male or female NOD.*Tnfsf10*$^{-/-}$ and WT NOD mice using the method described earlier. Freshly isolated islets were rested O/N in a 6cm bacterial petri dish in 3ml warm complete CMRL media. Healthy, islets were then handpicked and plated in a 96 well flat-bottomed plate @ 35islets per well in 150µl fresh warm CMRL media supplemented with 10% FCS, 2mM glutamine, filter sterilised Pen/Strep. Islet cells were stimulated with LPS 1mg/mL (serotype 0111:B4, Sigma) for 48 hours in a 5%CO_2 incubator at 37^{0}C. IP-10 levels in the islet-culture supernatant was measured using an IP-10 FlowCytomix simplex kit (BenderMedSystems GmbH, Vienna, Austria) by flowcytometry.

3.6 Anatomical pathology

3.6.1 Assessment of insulitis development

The degree of insulitis was scored by blind histological analysis of hematoxylin/eosin (H&E) stained sections of pancreata from female and male NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2}, NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice. Pancreas were fixed in 10% neutral buffered formalin for 18h, alcohol dehydrated and paraffin embedded. Paraffin blocks were sectioned at three levels at least 60µ apart, and the 6µ sections were transferred onto Superfrost plus slides. Slides were deparaffinised, rehydrated and then stained with H&E. 10 to 100 islets were

scored from each pancreas. The proportion of islets exhibiting each degree (scale 0–5) of insulitis was determined and expressed as a percentage of the total number of islets scored. Grade 0 represents no infiltrate, scale 1 represents periislet accumulation of mononuclear cells (<25% infiltration), scale 2 represents circumferential accumulation of mononuclear cells (25-50% infiltration), scale 3 represents 50-75% intraislet infiltration, scale 4 represents severe structural derangement and complete loss of beta cells, and scale 5 is diabetic.

3.7 Clinical pathology

3.7.1 Assessment of diabetes development

Random blood glucose (RBG) estimations of mouse peripheral blood were recorded every two weeks from the 8th to the 35th week for all mice included in the diabetes incidence study. RBG estimations in mice treated with BCG or saline were initially recorded on the day of treatment, and thereafter it was monitored fortnightly from 12-35 week of age. CY and SZ induced diabetes onset was monitored by RBG estimations performed every alternate day from the day of injection, until 30 and 50 days respectively. Adoptive transfer of diabetes was monitored by RBG estimations, thrice a week, starting 1-week post injection. Mice were reported diabetic if two blood glucose readings > 11.1mM were obtained, or one reading > 11.1mM was followed by otherwise unexplained disease. All the blood glucose estimations were performed on retro-orbital venous blood samples

by the glucose oxidase technique using CareSens™ blood glucose monitoring system (i-SENS Inc. Seoul, Korea).

3.7.2 Assessment of anaemia

After BCG or PBS injections the mice were bled at monthly intervals by retro-orbital venepuncture. Whole blood haemoglobin levels were determined by the sodium nitrite/azide method using a HemoCue Hb 201+ (HemoCue AB, Ängelholm, Sweden). Haematocrits were determined by centrifuging the blood in heparinised capillary tubes (Becton Dickinson, Parsippany, NJ) at 1000 g for 30min. After removing the plasma, direct Coombs tests were performed by washing the packed red cells three times in MACS buffer. 5µl of the washed red cells were resuspended in 1ml of the same buffer. Neat and five serial dilutions (1 in 100) of each sample were then incubated in round-bottom microtiter plates with 10µl of 5µg/ml polyclonal goat anti-mouse IgG (Sigma-Aldrich, St. Louis, USA) for 3 h at 37^0C. Wells in which the cells collected in a button were reported negative, while those with any signs of agglutination were recorded as positive. Red cells from female NOD/Lt mice> 500 days of age, which spontaneously developed haemolytic anaemia, served as positive controls.

3.7.3 Detection of antinuclear autoantibodies by Immunofluorescence

30µl of diluted sera (1:10 in PBS) were incubated for 30 min at room temperature on HEp-2 slide monolayers (ImmuGlo™, IMMCO Diagnostics, Inc.

Buffalo, NY) in a moistened incubation chamber. Slides were washed in PBS and incubated with 20μg/ml human adsorbed FITC conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, USA) for 40 min. Slides were then washed thrice with PBS and the presence of antinuclear autoantibodies (ANA) was detected by fluorescence microscopy. Positive control sera were taken from female NOD/Lt mice> 500 days of age, previously demonstrated to produce ANAs. Relative levels of ANAs were not titrated. Samples were declared as either positive (staining) or negative (no staining).

3.7.4 Detection of IC deposition by direct Immunofluorescence

Glomerular immune complex depositions in NOD mice were detected by immunohistochemical findings (deposition of C3c). Kidneys for immunofluorescence studies were snap frozen in Tissue-Tek O.C.T. compound (Miles Inc., Bloomington, IN) and stored at -70°C. Five μm cryostat sections were fixed in acetone, and direct immunofluorescence staining performed using tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse immunoglobulin M (IgM), IgG and IgA antibodies (Southern Biotechnology, Birmingham) and anticomplement C3c conjugated to fluorescein isothiocyanate (FITC; Nordic Immunology, Tilburg, the Netherlands). After rehydrating the sections in PBS, they were treated with 10% normal horse serum for 20 min, excess serum was blotted from around the sections, and the appropriate antibodies added. The samples were incubated in humidified chambers at room

temperature for 30 min, washed three times for 5 min in PBS, mounted and examined using a fluorescence microscope.

3.7.5 Estimation of cytokine production

Cytokine levels in the whole blood plasma were determined by bead based multiple analyte detection system, including, CBA Mouse flex sets (IL-3, IL-9, IL-12p70, IL-13, KC, MCP-1, MIG, MIP1a, MIP1b and RANTES), mouse Th1/Th2 cytokine kit (TNF, IFN-γ, IL-2, IL-4, IL-5), mouse inflammation kit (, IL-6, IL-10, MCP-1 TNF, IFN-γ and IL-12p70) all from BD Biosciences, San Diego, USA, and mouse Th1/Th2 10plex FlowCytomix Multiplex (GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α) from Bender MedSystems GmbH, Vienna. Capture beads (30 µl each, of respective analyte) together with 30 µl of mice blood plasma samples and 30 µl of PE detection reagent, were incubated in dark for 2 hour in a round-bottom microtiter plate. Beads were then washed twice with 200 µl of wash buffer, resuspended, and analysed using FACSCalibur or Cyan ADP flowcytometer. Cytokine standards were serially diluted and assayed as described above, to generate standard curves. Standard curves were generated and samples were quantified using the BD CBA software (BD Biosciences) or the FlowCytomix Pro 2.2 software (Bender MedSystems GmbH, Vienna, Austria). IP-10 levels in the islet-culture supernatant was measured using an IP-10 FlowCytomix simplex kit (BenderMedSystems GmbH, Vienna, Austria) according to the manufacturer's instructions on a FACSCalibur flowcytometer. Serial dilutions of the provided

cytokine standards were prepared and assayed to generate standard curves. The samples were quantified using the FlowCytomix Pro 2.2 software.

3.8 Statistical analysis

Data were analysed using GraphPad Prism software versions 5.0 and 6.0 for Mac/Windows. Results are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. In survival studies and flowcytometry experiments data was entered/exported into MS Excel and copied into GraphPad Prism. Depending on the sample size appropriate statistical tests were performed. Normality test was performed wherever sample size was more than 5 in each group, and depending on the outcome, a non-parametric or a parametric test was performed. $P< 0.05$ was deemed statistically significant. Statistical comparisons between treatments were calculated with one-way analysis of variance (ANOVA) using Dunn's multiple comparisons test. Fisher's exact test was used in the analysis of contingency tables, while Mann Whitney U-test was used for direct comparison of data sets. Survival distributions were analysed using Mantel-Cox log-rank test. A Mann Whitney U-test was undertaken to identify differentially regulated genes from the microarray data. A U-statistic of 0 was used as a conservative significance threshold; this value coincided with a lack of overlap in signal values between the two groups being compared.

.....

CHAPTER 4

.....

TRAIL MEDIATED PROTECTION OF DIABETES IN NOD MICE

4.1 Introduction

Members of Tumor Necrosis Factor (TNF) superfamily have been implicated in the destruction of pancreatic β-cells leading to the development of autoimmune diabetes in humans and in NOD mice (Chervonsky et al., 1997; Cheung et al., 2005; Ishizuka et al., 1999; Itoh et al., 1997). Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/APO2L/TNFSF10) is a typical member of the structurally related TNF superfamily. TRAIL is a type II transmembrane protein ligand for death receptors 4 (DR4/TRAILR1) and 5 (DR5/TRAILR2), decoy receptors 1 (TRAILR3) and 2 (TRAILR4) and Osteoprotegerin (Degli-Esposti et al., 1997; MacFarlane et al., 1997; Pan et al., 1997; Pitti et al., 1996; Sheridan et al., 1997; Walczak et al., 1997). TRAIL signalling through DR4/5 triggers multiple cell signals, including the recruitment of FADD and caspase-8, which subsequently lead to apoptosis via the extrinsic pathway with, or without the activation of Bid/Bax mediated intrinsic pathway (Suliman et al., 2001; Werner et al., 2002).

TRAIL is expressed on various cells of innate and adaptive immune system, including monocytes, macrophages, NK cells, DCs, activated T cells and B cells (Halaas et al., 2000; Wiley et al., 1995). Although TRAIL-deficient mice do not show signs of spontaneous autoimmunity, they are more susceptible to autoimmune diseases including experimental autoimmune encephalomyelitis

(EAE, an induced model of multiple sclerosis (Cretney et al., 2005; Hilliard et al., 2001)), an induced form of autoimmune arthritis (Lamhamedi-Cherradi et al., 2003b), autoimmune thyroiditis (Wang et al., 2005a) and autoimmune diabetes (Lamhamedi-Cherradi et al., 2003a).

Lack of TRAIL expression in TRAIL targeted mutant C57BL/6 mice enhanced autoimmune diabetes incidence and increased the degree of islet inflammation following multiple low-dose streptozotocin treatment (Lamhamedi-Cherradi et al., 2003b). The blockade of TRAIL signalling using a soluble receptor (sDR5) also exacerbated the onset of streptozotocin-induced diabetes (Lamhamedi-Cherradi et al., 2003a) and enhanced the adoptive transfer of the disease into NOD.*scid* mice by diabetogenic splenocytes from NOD mice (Mi et al., 2003), indicating a protective role for TRAIL in this disease. The mechanism(s) by which TRAIL signalling confers resistance to autoimmune diabetes in NOD mice is still unclear. Using the recently established targeted gene deficient mutants of *Tnfsf10* (NOD.*Tnfsf10*$^{-/-}$) mouse strain, this study aims to improve our understanding of the role of this death receptor ligand in the biology of β-cell destruction.

4.2 Results

4.2.1 Incidence and severity of diabetes in the TRAIL deficient NOD.*Tnfsf10*$^{-/-}$ mice.

Exacerbation of autoimmune diabetes in NOD mice as a result of TRAIL-signalling blockade by a soluble TRAIL receptor suggested a critical role for this ligand in autoimmune diabetes (Lamhamedi-Cherradi et al., 2003a). Adopting an alternative approach to validate the protective effect of TRAIL in diabetes pathogenesis, I used TRAIL deficient NOD mice with a targeted deletional mutation of *Tnfsf10* (NOD.*Tnfsf10*$^{-/-}$), generated in association with our collaborators Thomas Kay and Mark Smyth.

4.2.1.1 Spontaneous diabetes in TRAIL deficient NOD.*Tnfsf10*$^{-/-}$ mice.

To examine the effect of targeted deletion of TRAIL on the onset of type 1 diabetes in NOD/Lt mice, blood glucose levels were monitored in a cohort of 18 male and 21 female NOD.*Tnfsf10*$^{-/-}$ mice from 8 weeks to 32 weeks age, and the incidence of diabetes compared with that of male and female NOD/Lt mice (n=22 and 37 respectively, Figure 4.1). The genetic ablation of TRAIL in male NOD.*Tnfsf10*$^{-/-}$ mice trebled their incidence of spontaneous diabetes (NOD.*Tnfsf10*$^{-/-}$ 15/18 (83%) vs. NOD/Lt 6/22 (27%; p=0.0005, Fisher's exact test; $p < 0.0001$, life table analysis)). TRAIL deficiency did not have any significant effect on the incidence of spontaneous diabetes in female mice (NOD.*Tnfsf10*$^{-/-}$ 18/21 (86%) vs.

NOD/Lt 27/37 (73%)). The higher incidence of spontaneous diabetes in female NOD mice compared to their male littermates is a standard feature of the model and the higher penetrance of diabetes in female NOD mice could have masked any evidence of an enhancement of this trait.

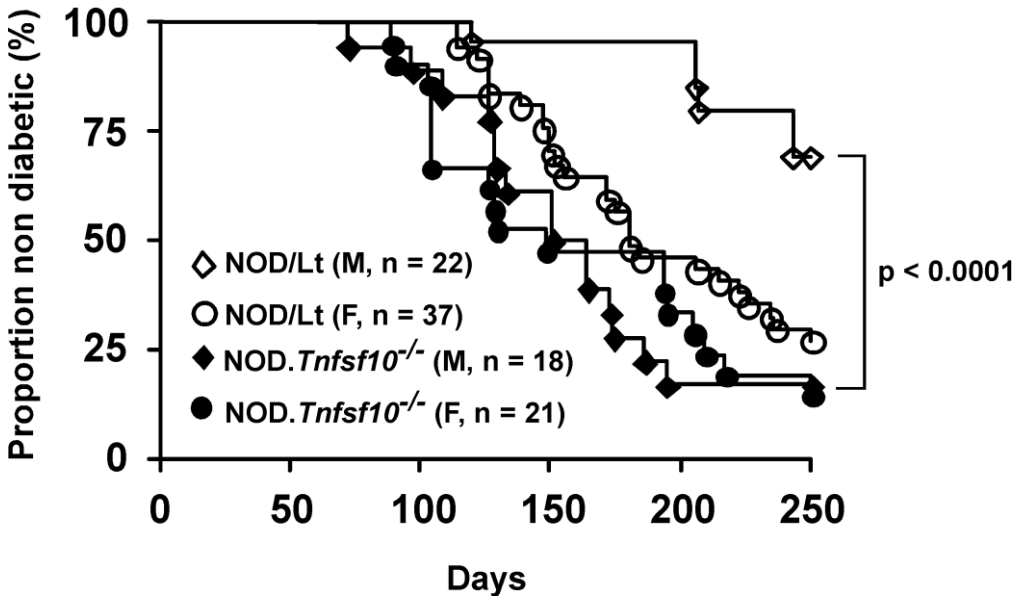


Figure 4.1 Spontaneous diabetes in TRAIL deficient NOD.*Tnfsf10*$^{-/-}$ mice. Incidence of spontaneous type 1 diabetes in male (diamonds) and female (circles) TRAIL deficient (black) and control NOD/Lt mice (white). Statistical comparison performed by life table analysis.

4.2.1.2 Cyclophosphamide-accelerated diabetes in NOD.*Tnfsf10*$^{-/-}$ mice.

Onset of autoimmune diabetes can be expedited in male and female NOD mice by treatment with the cytotoxic drug cyclophosphamide (CY), via a mechanism proposed to involve the inhibition or elimination of "suppressor cells" (Charlton et al, 1989). In order to determine the effect of TRAIL on CY accelerated diabetes, our collaborators Erika Cretney and Anabel Silva, staff at the Peter MacCallum institute, Melbourne, challenged pre-diabetic male and female NOD.*Tnfsf10*$^{-/-}$, NOD.*Tnfsf10*$^{+/-}$ and NOD.*Tnfsf10*$^{+/+}$ mice intraperitoneally (i.p.) with 200mg/kg Cyclophosphamide on day 0 and day 10. While all TRAIL deficient female mice (10/10) developed diabetes within 12 days of CY administration (Figure 4.2A), only 71% (5/7) of NOD.*Tnfsf10*$^{+/-}$ and 80% (8/10) of wild type littermates became diabetic ($p<0.0001$, life table analysis) in the study period. Furthermore, TRAIL deficiency enhanced the rate of onset of diabetes in male NOD.*Tnfsf10*$^{-/-}$mice, all of which become diabetic (10/10) while the disease developed in only 71% (5/7) of the NOD.*Tnfsf10*$^{+/-}$ and 80% (8/10) of wild type littermates (Figure 4.2b; $p<0.005$; life table analysis) during the study period. This suggests that the actions of cyclophosphamide and TRAIL ablation are at least additive, and that the role of TRAIL in inhibiting diabetes onset in NOD mice is not mediated via a cyclophosphamide-sensitive mechanism.

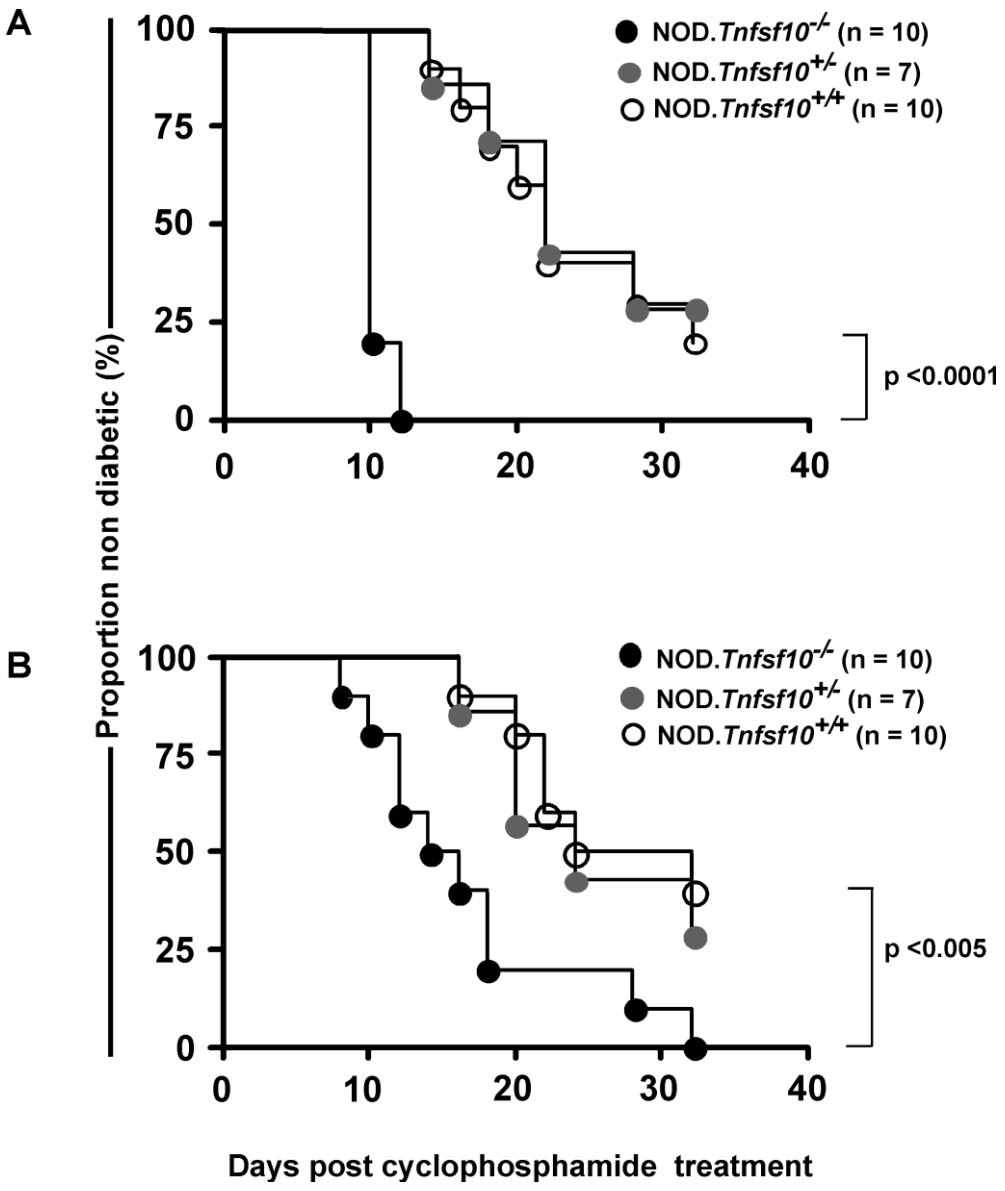


Figure 4.2 Cyclophosphamide-accelerated diabetes in TRAIL deficient NOD mice. Induction of diabetes in male (A) and female (B) NOD.*Tnfsf10*$^{-/-}$ (black), NOD.*Tnfsf10*$^{+/-}$ (grey) and NOD.*Tnfsf10*$^{+/+}$ (white) mice. Statistical comparison performed by life table analysis.

4.2.1.3 *M. bovis* exposure protects NOD.*Tnfsf10*$^{-/-}$ mice from diabetes

BCG (*M.bovis*) treatment provides close to total protection against diabetes in female NOD mice (Harada et al., 1990). *M.bovis* mediated suppression of insulitis and overt diabetes in NOD mice is dependent on the production of IFN-γ; *M.bovis* treated NOD.*Ifng*$^{-/-}$ mice were not protected (Serreze et al., 2001) – a result confirmed by our studies of targeted gene deficient mutant NOD. *Ifng* $^{-/-}$ (Figure 6.5). In light of studies that implicate IFN-γ in the upregulation of TRAIL on macrophages, DCs and NK cells in mice (Fanger et al., 1999; Griffith et al., 1999; Takeda et al., 2001), it was postulated that the predominantly proinflammatory cytokine milieu (e.g. IL-2, IL-12 and IFN-γ), generated in response to the *M.bovis* stimulation, induces TRAIL upregulation and its associated immunosuppressive effects in NOD mice. Diehl and colleagues reported an up-regulation in TRAIL expression and an enhancement of cytokine production in *M. bovis* stimulated macrophages from TRAIL receptor deficient mice (Diehl et al., 2004), suggesting a role for TRAIL signalling in the negative regulation of innate immune responses.

In order to test the hypothesis that TRAIL signalling contributed to the *M.bovis* mediated diabetes protection in NOD mice, 0.25 mg BCG was administered to 8 week old female NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice and the onset of diabetes monitored until 36 weeks of age by two-weekly random blood glucose determinations. TRAIL deficient NOD mice were protected from diabetes by BCG injection, albeit to a lesser extent than parental NOD/Lt mice (Figure 4.3). While

M.bovis treatment reduced the incidence of diabetes in NOD/Lt mice from 86% (12/14) to 21% (4/19; p<0.0002; life table analysis), genetic ablation of TRAIL moderately diminished the level of protection in NOD.*Tnfsf10*$^{-/-}$ mice, with a reduction of disease from 9/11 (82%) in the PBS treated group to 6/13 (46%; p<0.002; life table analysis) in the group that received BCG. This suggests that TRAIL signalling plays a limited role in diabetes protection mediated by BCG.

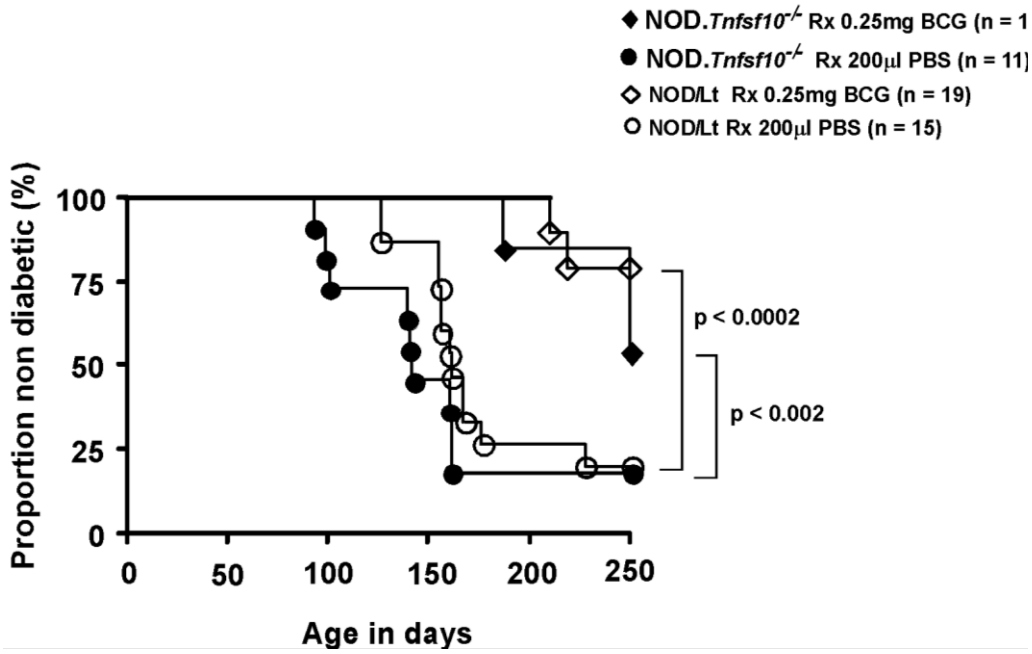


Figure 4.3 Protection from diabetes of NOD.*Tnfsf10*$^{-/-}$ mice by M.bovis exposure. Incidence of diabetes in PBS (circles) and heat-killed M.bovis treated (0.25mg i.v.; diamonds) NOD/Lt (white) and NOD. *Tnfsf10*$^{-/-}$ (black) mice. Statistical comparison performed by life table analysis.

4.2.2 TRAIL signaling in Intra-thymic negative selection and peripheral T cell regulation

The influence of TRAIL on autoimmunity was initially attributed to its role in antigen-induced thymic negative selection (Corazza et al., 2004; Lamhamedi-Cherradi et al., 2003b), a role that was later contested (Cretney et al., 2003; Diehl et al., 2004). As such a role would provide an explanation for the increased severity of spontaneous diabetes in male TRAIL-deficient NOD mice, the effects of the targeted deletion on endogenous mammary tumour virus (MTV) derived superantigen (SAGs) induced negative selection of Vβ3$^+$ and Vβ5$^+$ reactive thymocytes were studied. NOD mice bear *Mtv3* on chromosome 11, which is responsible for negative selection (deletion) of Vβ3$^+$ thymocytes (Fairchild et al., 1991; Tomonari et al., 1993). Similarly, it is likely that the NOD background contains either *Mtv8* or *Mtv9*, as the number of CD4$^+$ T cells bearing Vβ5 family TCR is comparatively low in the strain (Brenden and Bohme, 1999) and shows reduced proportions through thymic development.

4.2.2.1 Intra-thymic negative selection occurs normally in the absence of TRAIL signalling

The proportions of Vβ3 and Vβ5 cells in developmentally defined subsets of freshly isolated thymocytes from NOD/Lt and NOD.*Tnfsf10*$^{-/-}$ mice were defined by flowcytometry using a panel of antibodies (viz. anti- CD4, CD8, CD24, CD69, Vβ3/Vβ5 and βTCR). Within the CD4$^+$CD8$^+$CD24$^+$CD69^{hi}, CD4$^+$CD24$^+$CD69^{hi},

$CD4^+CD24^+CD69^{lo}$, $CD8^+CD24^+CD69^{lo}$, $CD4^+CD8^+CD24^+CD69^{hi}$, $CD4^+CD24^+CD69^{hi}$, $CD4^+CD24^+CD69^{lo}$, $CD8^+CD24^+CD69^{hi}$ and $CD8^+CD24^+CD69^{lo}$ subsets, the proportions of total βTCR^+ cells expressing Vβ5 and Vβ3 were determined. No significant differences in numbers or proportions of $V\beta5^+$ T cells (Figure 4.4) and $V\beta3^+$ T cells (Figure 4.5) were found between male NOD.*Tnfsf10*$^{-/-}$ and parental male NOD/Lt mice; consistent with the hypothesis that TRAIL-receptor signalling is not involved in the thymic selection process. Similar results were obtained studying the effects of targeted ablation of TRAIL on $V\beta5^+$ and $V\beta3^+$ T cell proportions in thymocytes from female mice (Figures 4.6 and 4.7). It is therefore unlikely that differences in thymic deletion are responsible for the dissimilarity observed in the incidence of diabetes in NOD.*Tnfsf10*$^{-/-}$ mice.

4.2.2.2 T cell subsets in NOD.*Tnfsf10*$^{-/-}$ mice

Although the above results suggest a limited role for TRAIL in thymic tolerance induction in the context of autoimmune diabetes, its function in regulating peripheral T cell autoreactivity needs to be investigated. It is likely that T cells primed by antigens shed during the physiological wave of β-cell death, initiate this autoreactivity (Trudeau et al., 2000; Turley et al., 2003). The potential for TRAIL to induce apoptosis in islet-antigen activated T cells was evident in a tolerance model developed by Griffith and colleagues (Griffith et al., 2007). Induction of the death receptor ligand appeared to inhibit the expansion of antigen-reactive $CD4^+$ T cells.

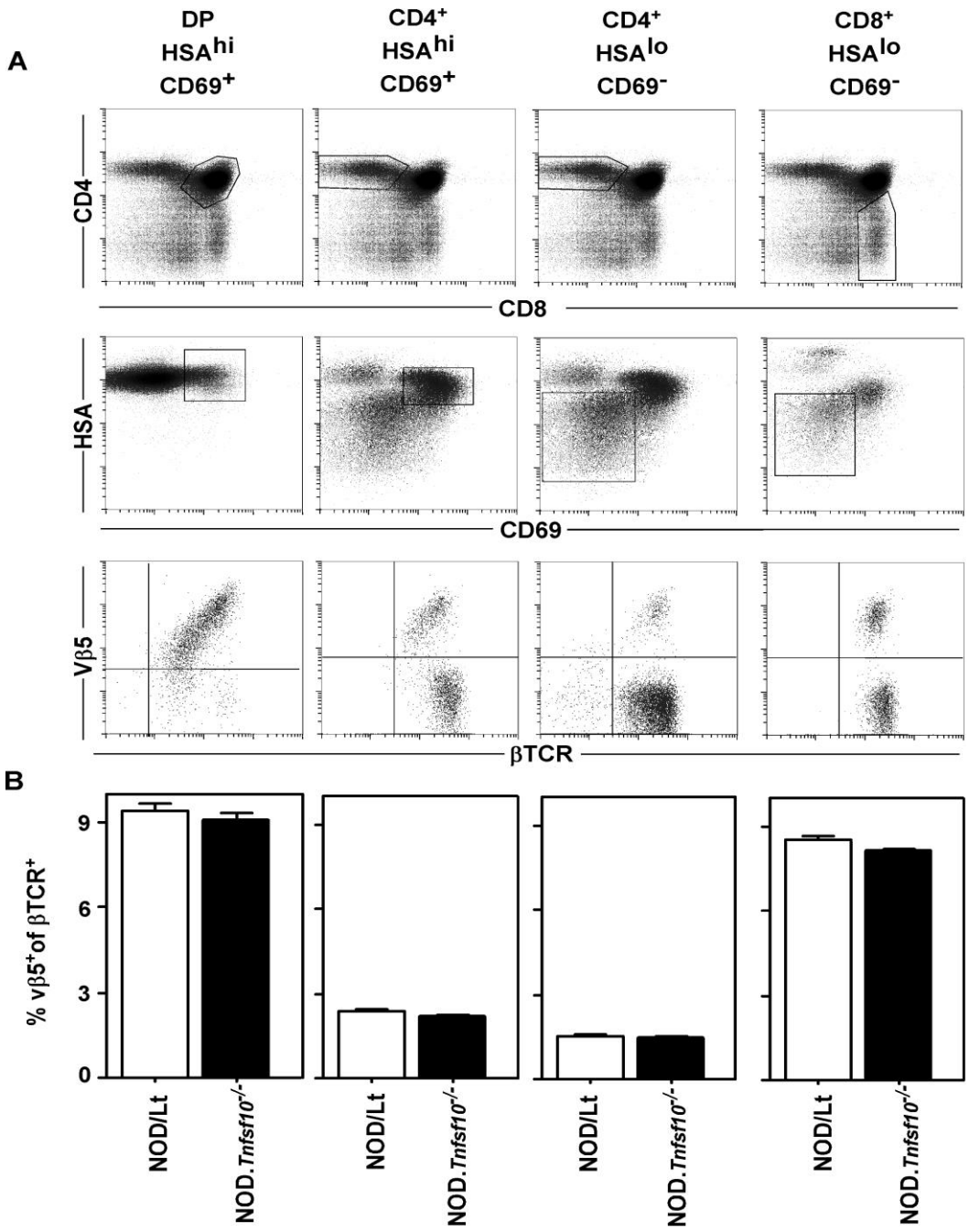


Figure 4.4 Vβ5 proportions in male NOD mice. Proportions of total βTCR$^+$ thymic cells expressing Vβ5 amongst various developmental subsets from NOD.*Tnfsf10*$^{-/-}$ (n=8; black) and NOD/Lt (n =8; clear) mice applying a gating strategy (A) based on CD4, CD8, HSA and CD69 expression. The ratio of vβ5$^+$ to total βTCR$^+$ cells is represented by histogram (B).

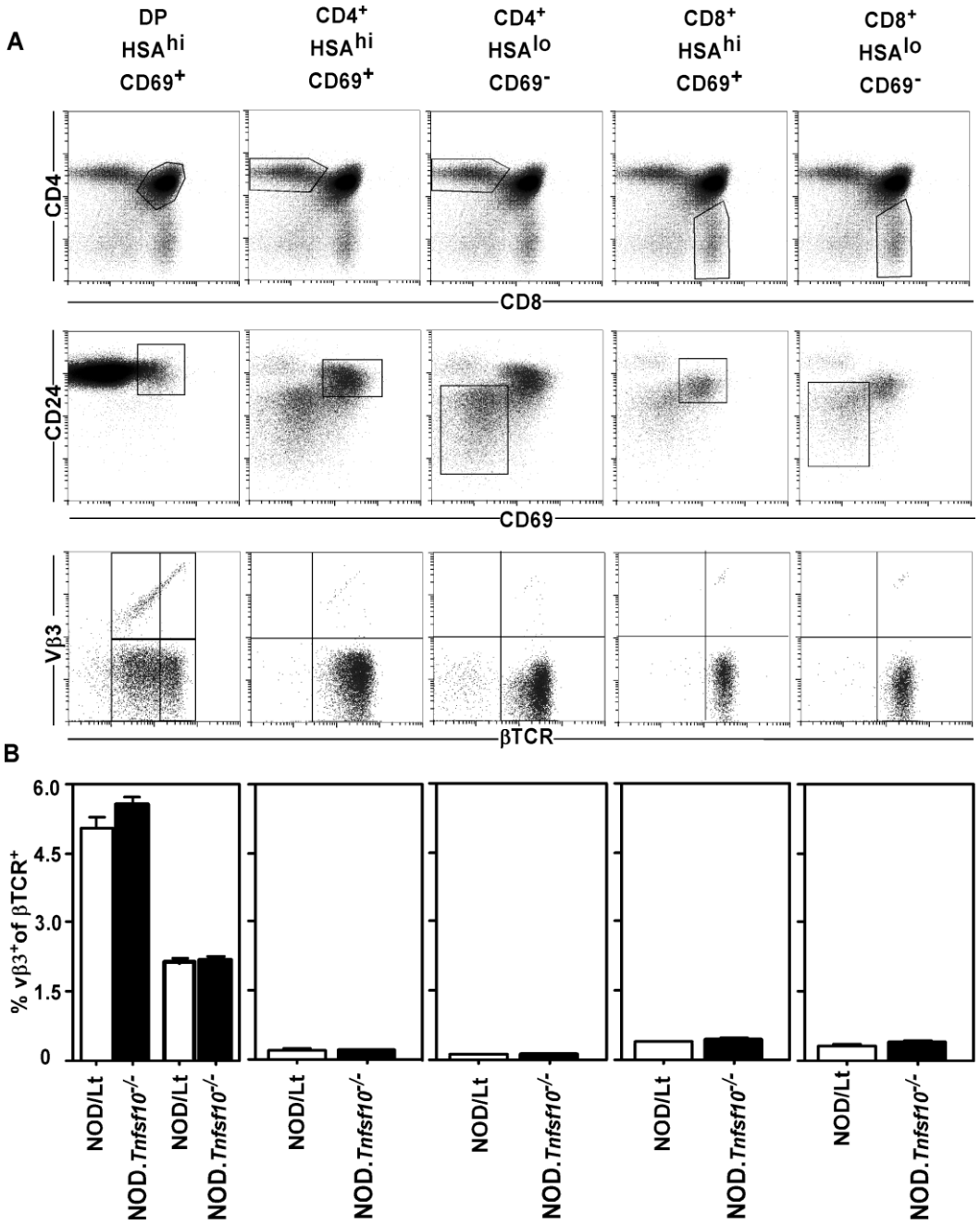


Figure 4.5 Vβ3 proportions in male NOD mice. Proportions of total βTCR$^+$ thymic cells expressing Vβ3 amongst various developmental subsets from NOD.*Tnfsf10*$^{-/-}$ (n=8; black) and NOD/Lt (n =8; clear) mice applying a gating strategy (A) based on CD4, CD8, HSA and CD69 expression. The ratio of Vβ3 $^+$ to total βTCR$^+$ cells is represented by histogram (B).

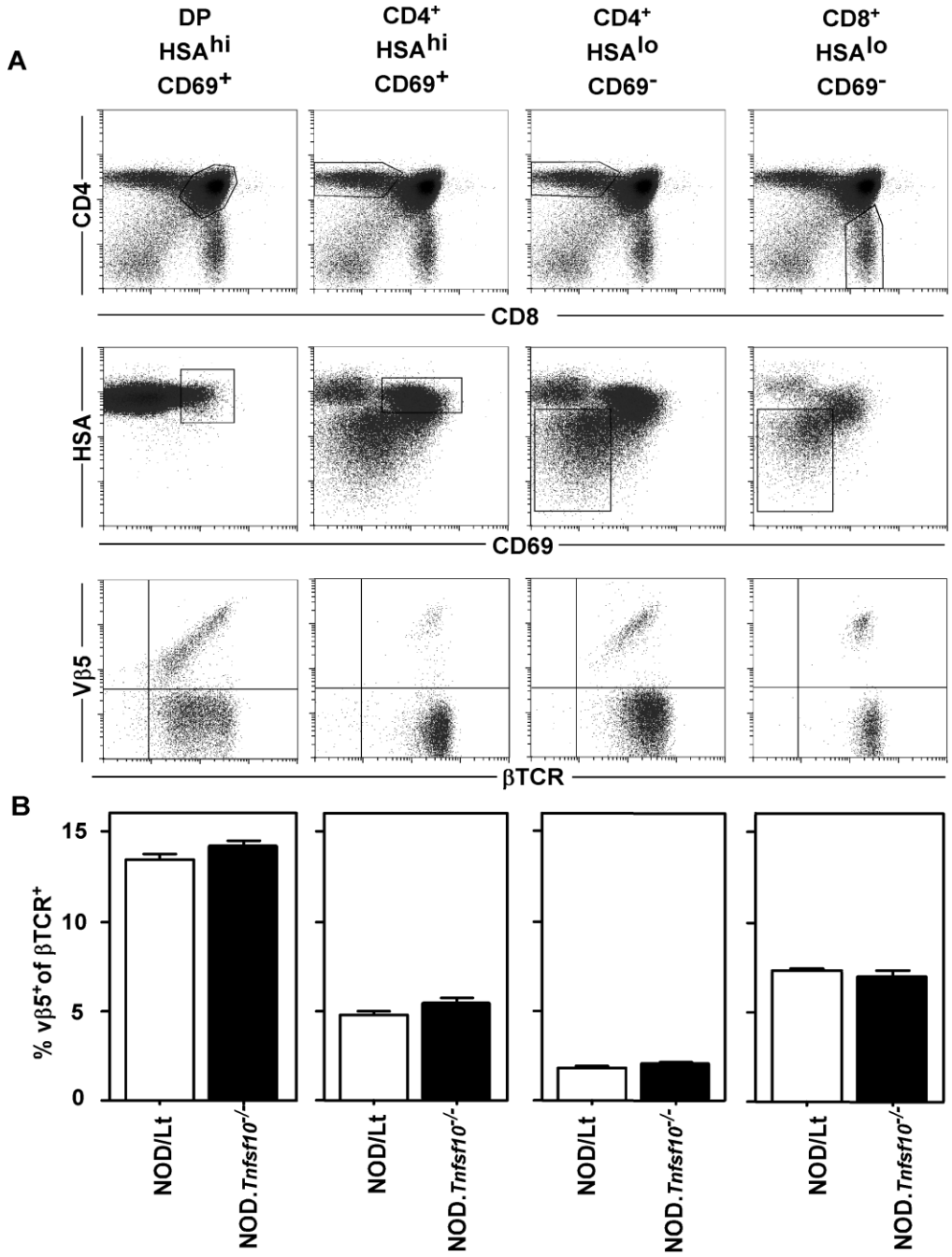


Figure 4.6 Vβ5 proportions in female NOD mice. Proportions of total βTCR$^+$ thymic cells expressing Vβ5 amongst various developmental subsets from NOD.*Tnfsf10*$^{-/-}$ (n=8; black) and NOD/Lt (n =8; clear) mice applying a gating strategy (A) based on CD4, CD8, HSA and CD69 expression. The ratio of vβ5$^+$ to total βTCR$^+$ cells is represented by histogram (B).

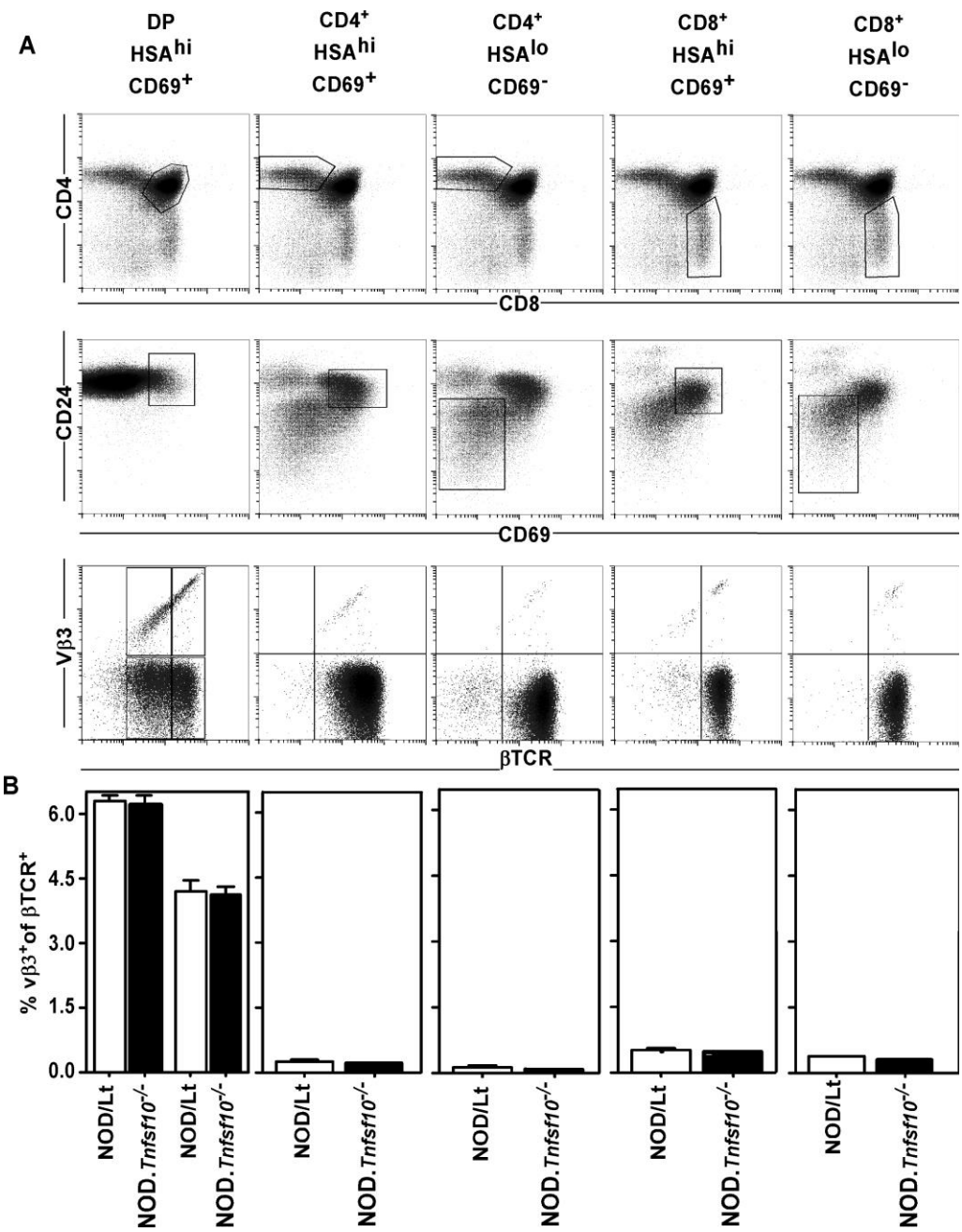


Figure 4.7 Vβ3 proportions in female NOD mice. Proportions of total βTCR$^+$ thymic cells expressing Vβ3 amongst various developmental subsets from NOD.*Tnfsf10*$^{-/-}$ (n=8; black) and NOD/Lt (n =8; clear) mice applying a gating strategy (A) based on CD4, CD8, HSA and CD69 expression. The ratio of Vβ3 $^+$ to total βTCR$^+$ cells is represented by histogram (B).

Moreover, the detection of functional TRAIL-R expression on human (Herbeuval et al., 2005)and mouse (Zhang et al., 2003) activated T cells, but not on resting T cells, suggest that TRAIL could play a role in induction of peripheral tolerance.

In order to test the inhibitory role of TRAIL on the proliferation and survival of activated T cells, the proportions of activated ($CD4^+CD44^+CD62L^-$), memory ($CD4^+CD44^{int}CD62L^{int}$) and naïve ($CD4^+CD44^{low}CD62L^+$) phenotype $CD4^+$ and $CD8^+$ T cells in the spleens, livers and pancreatic lymph nodes of NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice were examined. Flow cytometric analyses failed to identify any robust differences after correction for multiple hypotheses testing (Bonferroni multiple comparison test, Figure 4.8). Similar results were obtained analysing T cell subsets of female NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice (Figure 4.9).

4.2.3 Adoptive transfer of diabetes by TRAIL deficient diabetogenic splenocytes

To determine whether the increased proportions of memory $CD8^+$ T cells in spleens of NOD.*Tnfsf10*$^{-/-}$ mice was of functional significance, adoptive transfer of diabetogenic splenocytes from NOD.*Tnfsf10*$^{-/-}$ and parental NOD/Lt mice, into 8-10 week old female NOD.*scid* recipients was performed. There was no difference in the diabetogenic activity of splenocytes from male (Figure 4.10A) or female (Figure 4.10B) mice of either strain, suggesting no role for TRAIL in controlling T cell expansion and/or activation.

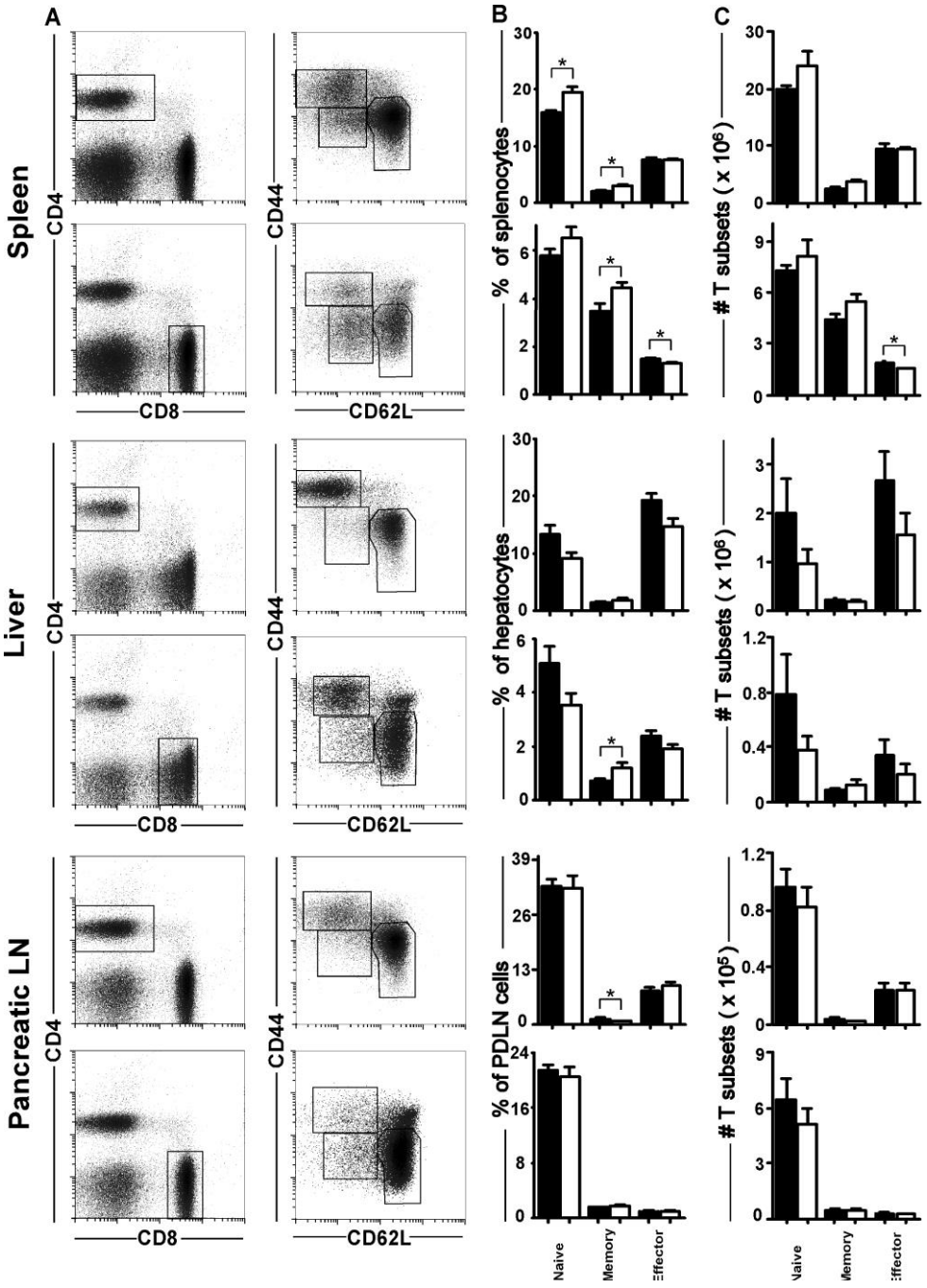


Figure 4.8 Peripheral T cell subsets in male NOD.*Tnfsf10*$^{-/-}$ mice. Representative plots of proportions of CD4^{+}CD44^{low}CD62L^{+} (naïve), CD4^{+}CD44^{+}CD62L^{-} (effector) and CD4^{+}CD44^{int}CD62L^{int} (memory) T cells from spleens, livers and pancreatic lymph nodes of male NOD.*Tnfsf10*$^{-/-}$ (n=5; black filled) and NOD/Lt mice (n =5; clear) (A). T cell subset proportions (mean ± SEM) and absolute numbers (mean ± SEM) represented by histograms (B and C respectively). Significance by Mann-Whitney U test; values are not corrected for multiple hypothesis testing. * represents $p < 0.05$.

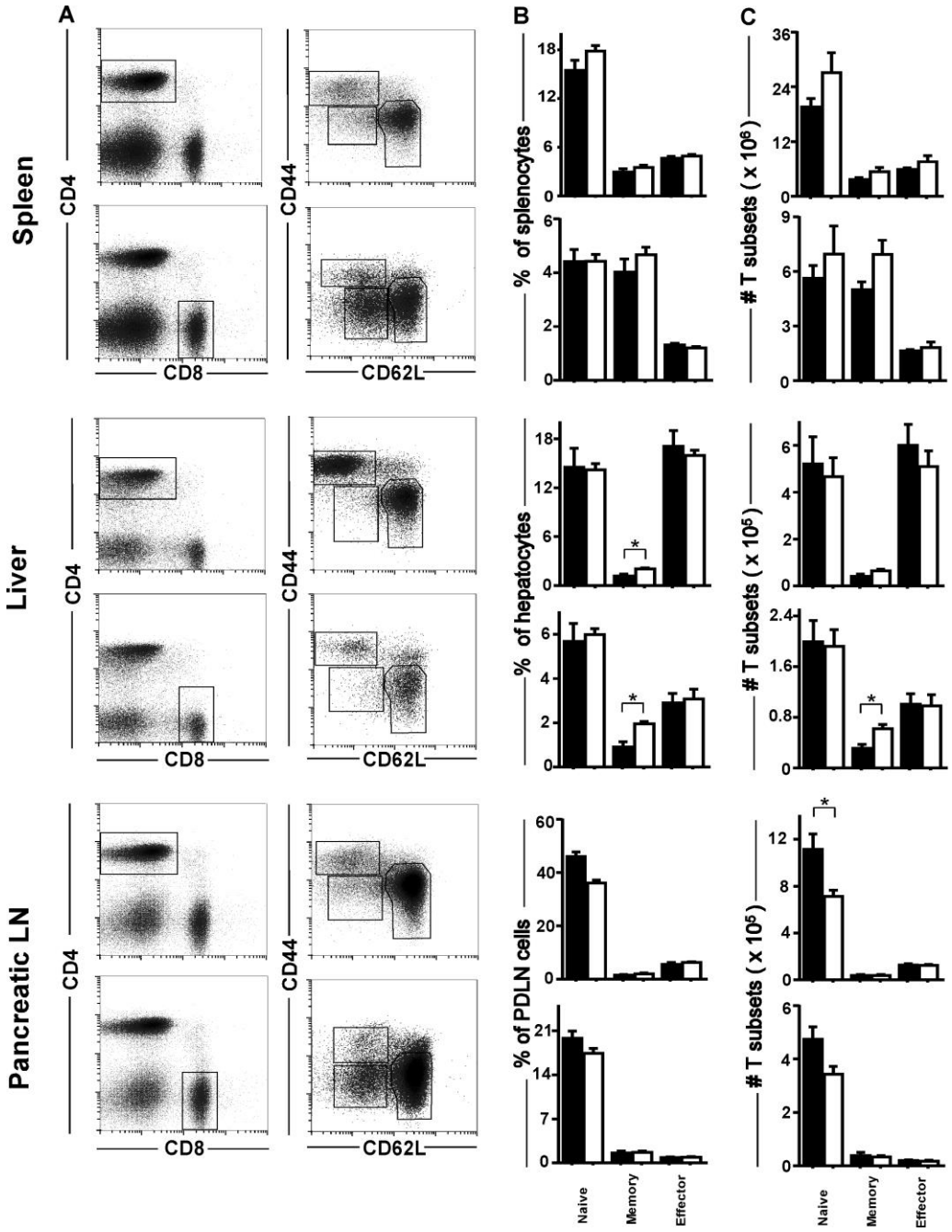


Figure 4.9 Peripheral T cell subsets in female NOD.*Tnfsf10*$^{-/-}$ mice. Representative plots of proportions of CD4^{+}CD44^{low}CD62L^{+} (naïve), CD4^{+}CD44^{+}CD62L^{-} (effector) and CD4^{+}CD44^{int}CD62L^{int} (memory) T cells from spleens, livers and pancreatic lymph nodes of male NOD.*Tnfsf10*$^{-/-}$ (n=5; black filled) and NOD/Lt mice (n =5; clear) (A). T cell subset proportions (mean ± SEM) and absolute numbers (mean ± SEM) represented by histograms (B and C respectively). Significance by Mann-Whitney U test; values are not corrected for multiple hypothesis testing. * represents $p < 0.05$.

This result raised the questions whether the enhanced beta cell destruction seen in the mutant mice was a characteristic of the responding T cells, or whether it was even a haematopoietic cell-intrinsic trait? To examine these questions, reciprocal spleen cell transfers into irradiated recipients were performed between NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice. On transferring TRAIL deficient diabetogenic splenocytes into irradiated male NOD.*Tnfsf10*$^{-/-}$(n=5) and parental NOD/Lt (n=8) mice, all the TRAIL deficient recipients developed diabetes within 3 weeks, while a significant delay in diabetes onset (> 14 weeks) and a decrease in proportion (6/8) occurred in the wild type recipients ($p<0.0003$; life table analysis, Figure 4.11A). Similar results were observed when diabetogenic wild type splenocytes were transferred into irradiated NOD.*Tnfsf10*$^{-/-}$(n=6) and NOD/Lt (n=10) recipients. Whereas 6/6 TRAIL deficient mutants became diabetic within 5 weeks, the disease was manifested in only 5/10 NOD/Lt recipients, in the course of 14 weeks post transfer ($p<0.0003$; life table analysis; Figure 4.11A). A significant difference in the rate of onset of diabetes in irradiated female recipients also occurred following the adoptive transfer of splenocytes from diabetic female TRAIL KO mice. While all TRAIL deficient female recipients (n=3) became diabetic within 2 weeks, the onset of disease in the parental NOD/Lt mice (n=4) was delayed by 8 weeks ($p<0.02$, life table analysis, Figure 4.11B). Similarly, following transfer of diabetogenic wild type splenocytes into irradiated NOD.*Tnfsf10*$^{-/-}$ (n=5) and NOD/Lt (n=3) recipients, all the TRAIL deficient mutants became diabetic within 3 weeks, while there was a significant delay (>14 weeks) before diabetes

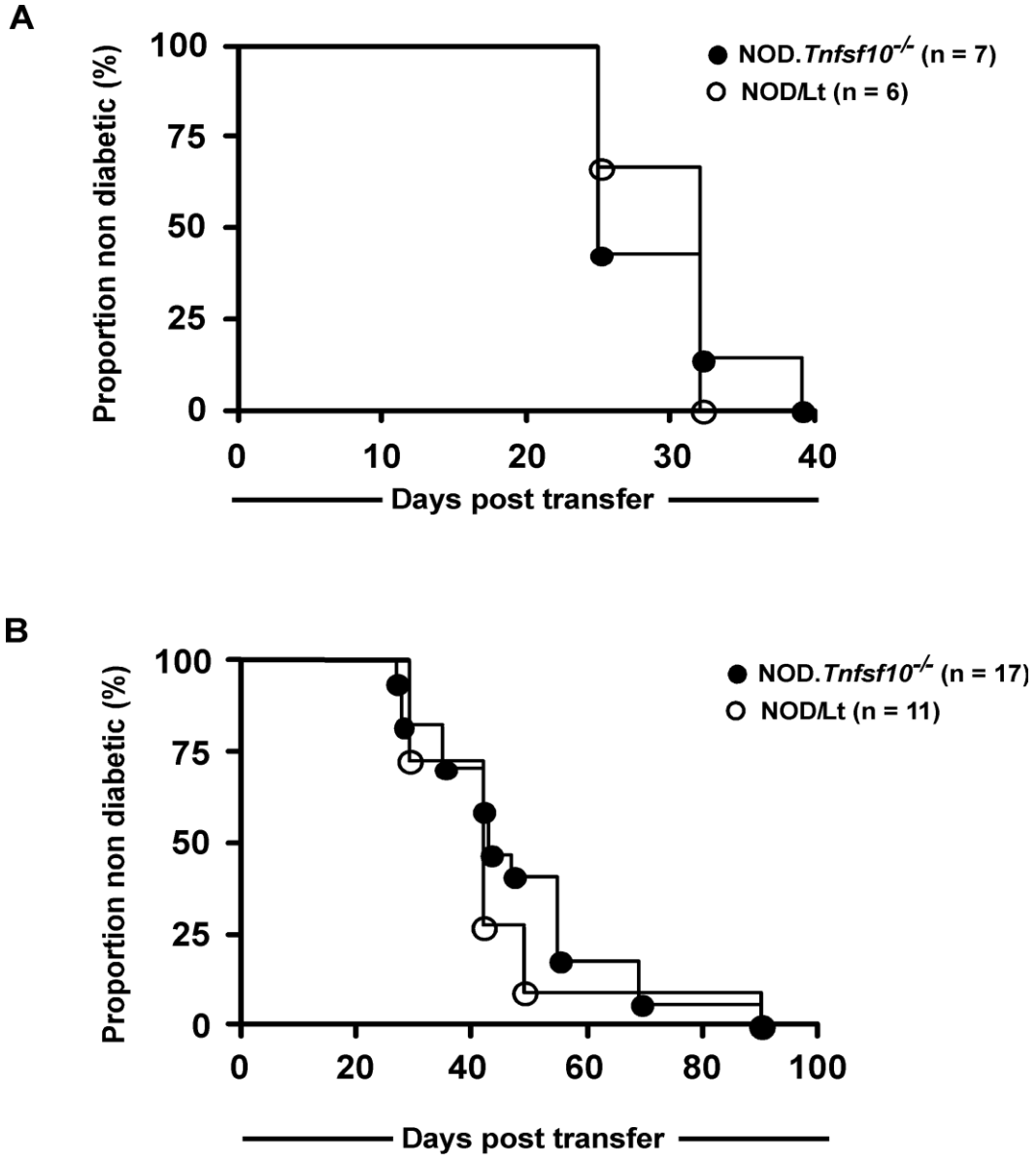


Figure 4.10 Adoptive transfer of diabetogenic splenocytes into NOD.*scid* mice. Adoptive transfer of diabetes into 8-10 week old female NOD.scid mice from male (A) or female (B) NOD/Lt (clear) or NOD.*Tnfsf10*$^{-/-}$ (solid) mice.

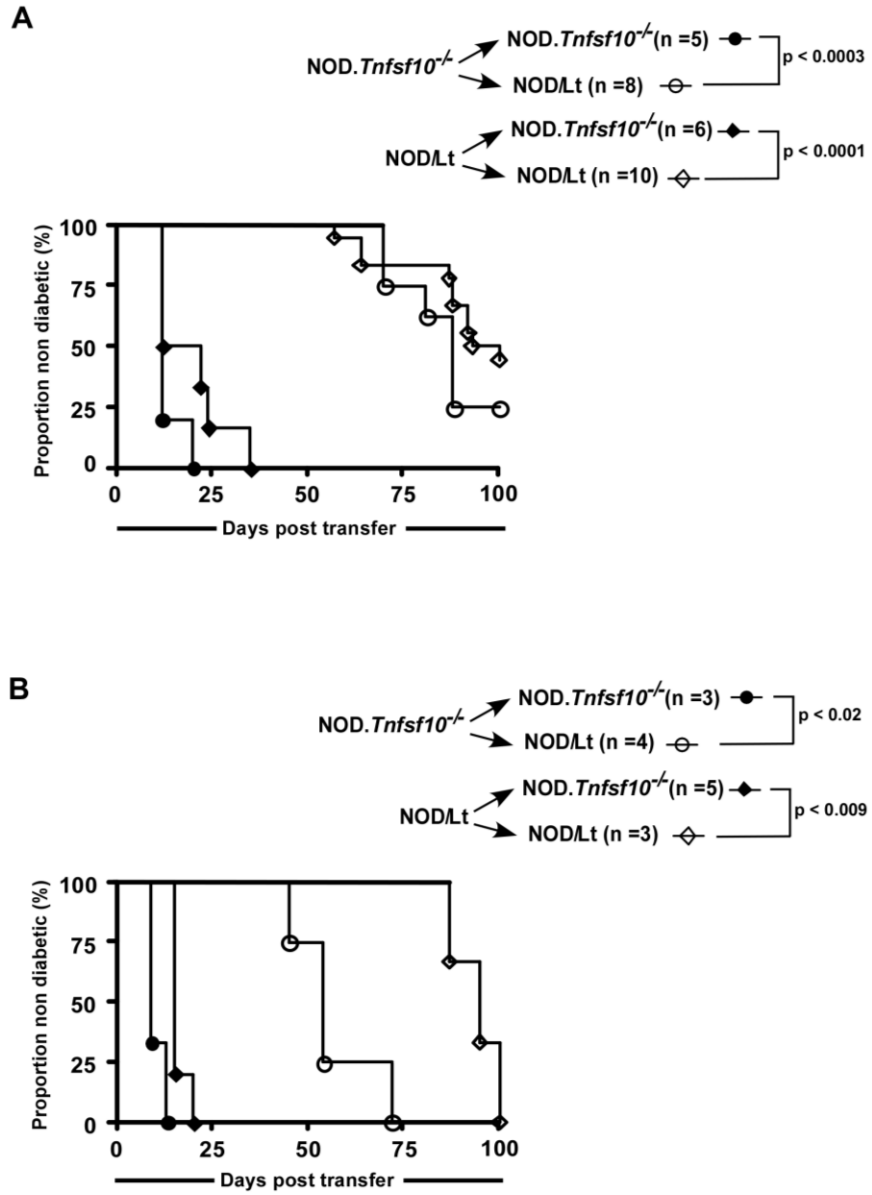


Figure 4.11 Reciprocal spleen cell transfers into irradiated male (A) and female (B) recipients. Diabetes development following reciprocal transfer of splenocytes between TRAIL-deficient (solid) and control NOD mice (clear). Pooled splenocytes from diabetic NOD.*Tnfsf10*$^{-/-}$ mice transferred into irradiated NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt (solid circles and clear circles respectively); and splenocytes from diabetic NOD/Lt into NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt (solid diamonds and clear diamonds respectively). Statistical comparison performed by life table analysis.

occurred in the parental NOD/Lt recipients ($p<0.009$; life table analysis; Figure 4.11B). To summarise, regardless of whether the experiments were performed in male or female mice, accelerated diabetes was associated with TRAIL-deficient recipients, not donors, raising the possibility that TRAIL plays some previously unidentified role in immune regulation, resulting in diabetes suppression in wild type NOD mice.

Although the adoptive transfer experiments indicated that the major variable affecting outcome was the genotype of the recipient (Figures 4.11A and B), dose titration of the diabetogenic splenocytes transferred revealed slight but significant differences in the autoimmune potential of TRAIL deficient cells. Diabetes occurred rapidly in all cohorts of irradiated male NOD.*Tnfsf10*$^{-/-}$ mice that received $20x10^6$, $16x10^6$, $12x10^6$, $8x10^6$ or $5x10^6$ diabetogenic TRAIL deficient splenocytes (Figure 4.12A). However the rate of disease induction by the lowest dose ($5x10^6$ cells) was slightly delayed compared to the highest dose of $20x10^6$ cells ($p<0.05$; life table analysis). A similar titration of male NOD/Lt splenocytes transferred into same sex irradiated NOD.*Tnfsf10*$^{-/-}$ recipients revealed a significant disparity in the autoimmune potential of the transferred cells (Figure 4.12B). While the transfer of 20 million splenocytes could induce the disease in within 4-5 weeks, disease induction was delayed in case of the lowest dose (5 million splenocytes; $p<0.02$; life table analysis), recipients of which became diabetic during the course of 14 weeks post transfer. Rapid onset of diabetes was noted in irradiated female

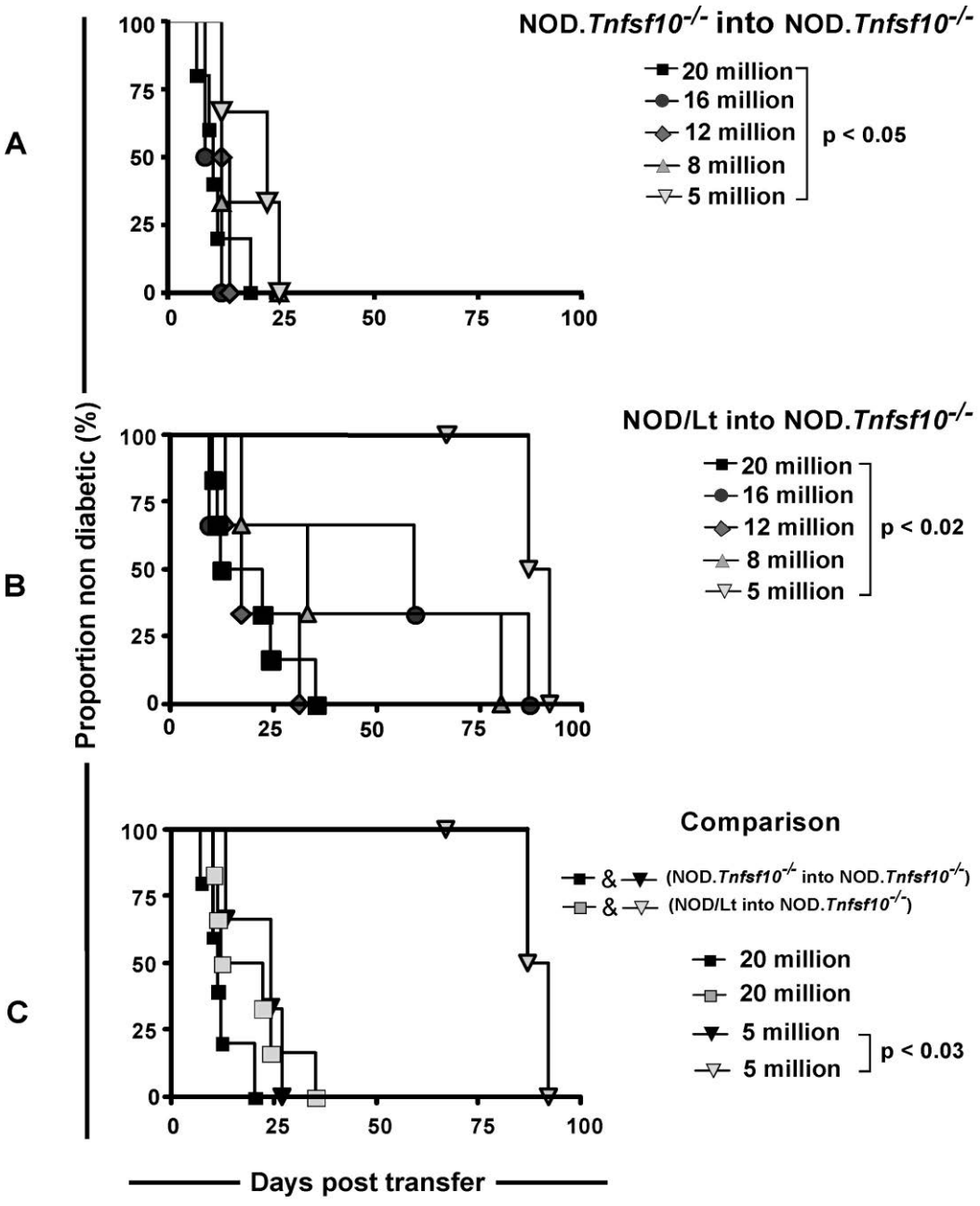


Figure 4.12 Dose titration of the autoimmune potential of male NOD.*Tnfsf10*$^{-/-}$ splenocytes. Dose titration of the autoimmune potential of splenocytes adoptively transferred from diabetic NOD.*Tnfsf10*$^{-/-}$ (A) and NOD/Lt (B) mice into irradiated male NOD.*Tnfsf10*$^{-/-}$ recipients. Every dose had 3 recipients or more. Comparison of the autoimmune potential of the highest (square) and lowest (inverted triangles) doses of diabetogenic splenocytes from NOD.*Tnfsf10*$^{-/-}$ (black) and NOD/Lt (grey), transferred into a TRAIL deficient environment (C). Statistical comparison performed by life table analysis.

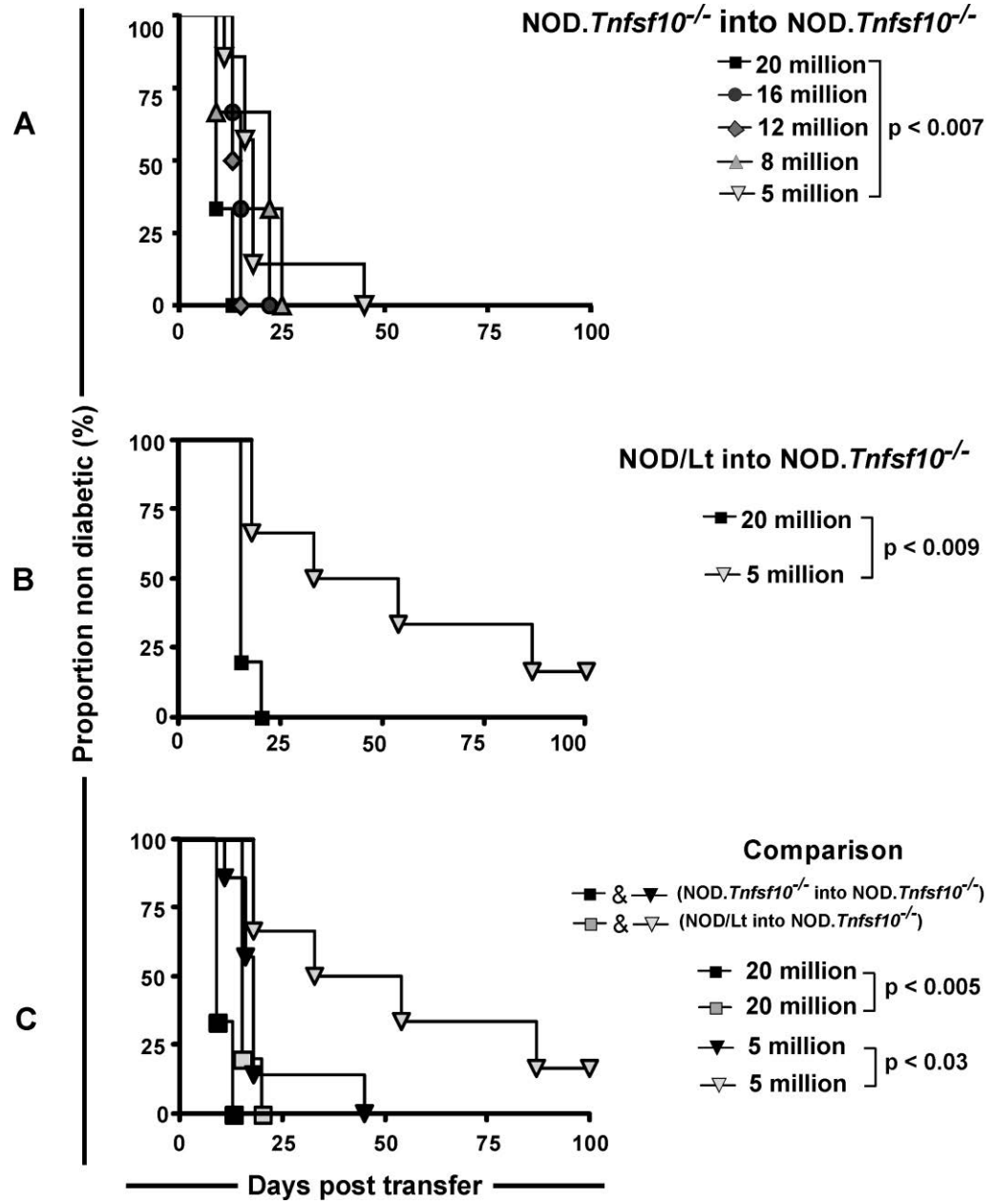


Figure 4.13 Dose titration of the autoimmune potential of female NOD.*Tnfsf10*$^{-/-}$ splenocytes. Dose titration of the autoimmune potential of splenocytes adoptively transferred from diabetic NOD.*Tnfsf10*$^{-/-}$ (A) and NOD/Lt (B) mice into irradiated male NOD.*Tnfsf10*$^{-/-}$ recipients. Every dose had 3 recipients or more. Comparison of the autoimmune potential of the highest (square) and lowest (inverted triangles) doses of diabetogenic splenocytes from NOD.*Tnfsf10*$^{-/-}$ (black) and NOD/Lt (grey), transferred into a TRAIL deficient environment (C). Statistical comparison performed by life table analysis.

NOD.*Tnfsf10*$^{-/-}$ recipients following the adoptive transfer of $20x10^6$, $16x10^6$, $12x10^6$, $8x10^6$ or $5x10^6$ TRAIL deficient splenocytes from recently diabetic female NOD.*Tnfsf10*$^{-/-}$ mice (Figure 4.13A). However a significant disparity in the rate of disease induction was evident between the highest and the lowest dose transfers ($p<0.007$; life table analysis). The difference in the rate of disease induction in irradiated NOD.*Tnfsf10*$^{-/-}$ recipients was more dramatic with diabetogenic splenocytes transferred from female NOD/Lt mice (Figure 4.13B; $p<0.009$; life table analysis).

Summing up, the reciprocal transfer experiments revealed a higher incidence of diabetes in TRAIL deficient recipients (Figure 5.11A and 5.11B) but the adoptive transfers into NOD.s*cid* mice showed no difference in the diabetogenic activity of splenocytes from mice of either strain (Figure 5.10A and 5.10B), indicating that the diabetes phenotype of TRAIL deficient mice is not lymphoid cell intrinsic. The more sensitive titration experiments (especially the lowest dose), however, revealed slight differences in the diabetogenic activity of splenocytes bearing the targeted deletional mutation of *Tnfsf10.* (Figure 4.12C and 4.13C). The median survival time (MST) for male recipients of lowest dose of diabetogenic NOD and NOD.*Tnfsf10*$^{-/-}$ splenocytes were 89.5 days and 24 days respectively ($p<0.03$, life table analysis), while MST for female recipients were 43.5 days and 18 days respectively ($p<0.03$, life table analysis).

4.2.4 Streptozotocin induced diabetes

The accelerated diabetes associated with TRAIL-deficient recipients was suggestive of a protective effect of TRAIL signalling on the pancreatic β-cells in NOD/Lt mice. Streptozotocin is a toxin that induces superoxide radical and nitric oxide mediated pancreatic β-cell damage (Szkudelski, 2001). In order to determine if the diabetes protective effect of TRAIL was due to differences in susceptibility of pancreatic β-cells to cell death, parental NOD/Lt (n=4) and NOD.*Tnfsf10*$^{-/-}$ mice (n=4) were treated with 120mg/kg SZ. The highest recorded random blood sugar readings were similar in both the strains (Figure 4.14A) and all of them developed diabetes within the 2-week study period (Figure 4.14B). As NOD.*Tnfsf10*$^{-/-}$ mice were no more susceptible to SZ induced diabetes than the parental control strain, it is unlikely that the accelerated diabetes associated with NOD.*Tnfsf10*$^{-/-}$ mice is due to an increase in susceptibility to damage of TRAIL deficient islets. However, it needs to be mentioned that susceptibility of TRAIL deficient β-cells to immune effector mechanisms of killing were not tested while arriving at this conclusion.

4.2.5 Effect of TRAIL deficiency on numbers of immunoregulatory cells

Natural regulatory T cells (Tregs) and NKT cells are two potent immuno-regulatory T cell phenotypes involved in peripheral tolerance. A reduction in their numbers and/or functions is reported in autoimmune-prone mice (Baxter et al., 1997; Hammond et al., 2001; Piccirillo et al., 2005).

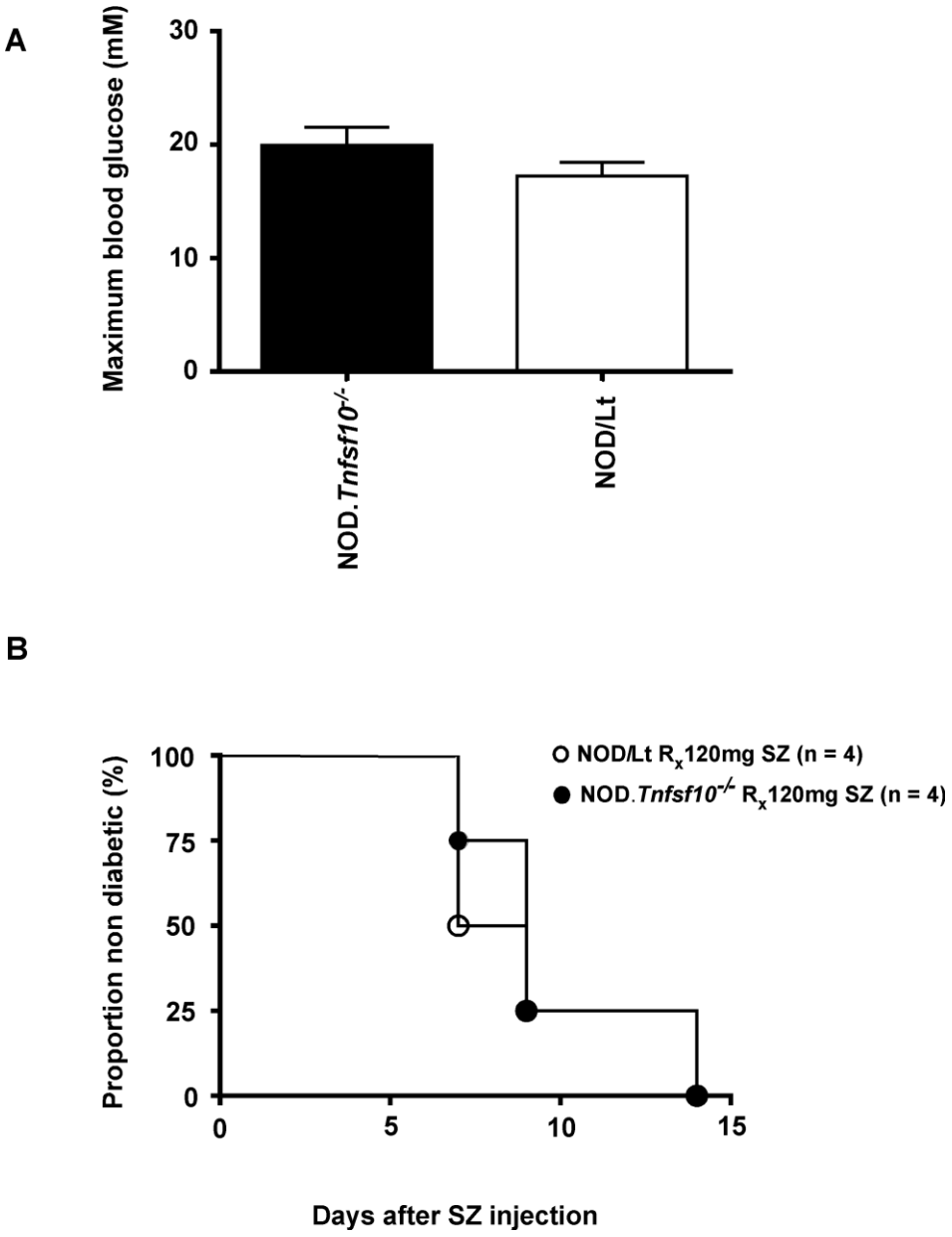


Figure 4.14 Maximum blood sugar levels and diabetes development following 120mg streptozotocin treatment of NOD.*Tnfsf10*$^{-/-}$ and control NOD/Lt mice. Highest recorded random blood sugar readings in male NOD/Lt (empty bar, n=4) and NOD.*Tnfsf10*$^{-/-}$ (solid bar, n=4) mice, post a 120mg/kg SZ treatment (A). Life table analysis of streptozotocin induced diabetes in NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice (B).

The TRAIL/DR5 pathway is one of the mechanisms proposed to be used by these immunosuppressive cells to regulate immune responses (Nieda et al., 2001; Ren et al., 2007). In order to determine whether genetic ablation of TRAIL in NOD.*Tnfsf10*$^{-/-}$ mice affected the numbers of TCRβ^{+}CD4^{+}CD25^{+}FoxP3^{+} immunoregulatory T cell population, flow cytometric analysis was performed on the thymi, spleens, livers and pancreatic lymph nodes of male NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice. The only significant difference identified was an increase in Treg proportions in the thymi of NOD.*Tnfsf10*$^{-/-}$ mice (6.95±0.31 % vs. 5.63±0.09 %; $p<0.0079$; Man-Whitney U test; Figure 4.15). This difference remained significant after correction for multiple hypotheses testing (Bonferroni multiple comparison test), but cannot explain the increased susceptibility of male NOD.*Tnfsf10*$^{-/-}$ mice to diabetes. Slight increases were identified in proportions of Treg in the thymi (4.76±0.15 % vs. 4.21±0.14 %; $p<0.0079$; Man-Whitney U test) and spleens (8.26±0.36 % vs. 6.08±1.05 %; $p<0.0079$; Man-Whitney U test) of female NOD. *Tnfsf10*$^{-/-}$ mice (not shown), but these differences did not withstand correction for multiple hypothesis testing (Bonferroni multiple comparison test).

On comparing the numbers of NKT cells in the thymi and peripheral lymphoid organs of TRAIL deficient NOD mice with those of wild type NOD/Lt by flow cytometry, male NOD.*Tnfsf10*$^{-/-}$ mice were found to have significantly higher proportions of alphaGalCer/CD1d-tetramer^{+} NKT cells in their thymi (0.41±0.04 % vs 0.21±0.03 %; $p<0.002$; Man-Whitney U test). However, in the periphery,

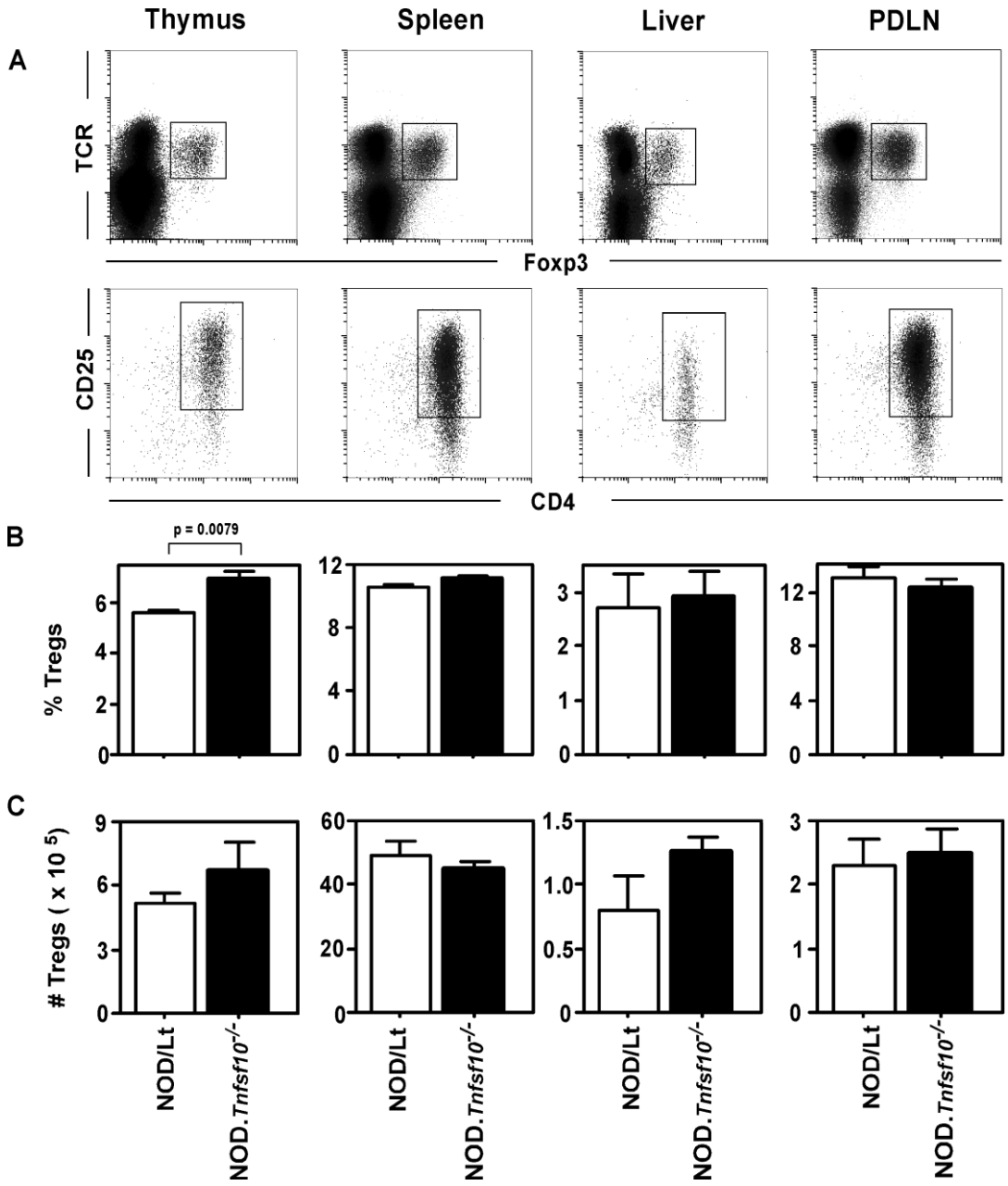


Figure 4.15 Regulatory T cells in male NOD.*Tnfsf10*$^{-/-}$ mice. Representative plots of proportions of TCR^{+}CD4^{+}CD25^{+}FoxP3^{+} Tregs in thymi, spleens, livers and pancreatic lymph nodes of male NOD.*Tnfsf10*$^{-/-}$ (n=5) and NOD/Lt (n=5) mice. Treg proportions (mean ± SEM) and absolute numbers (mean ± SEM) represented by histograms (B and C respectively). Significance by Mann-Whitney U test.

a significant difference was observed only in the livers (15.99±1.44 % vs 10.2±1.36 %; p<0.04; Man-Whitney U test; Figure 4.16). Similar observations were made in the thymi of female NOD.*Tnfsf10*$^{-/-}$ mice, which had significantly higher proportions of NKT cells (0.32±0.02 % vs 0.18±0.02 %; p<0.001; Man-Whitney U test). Among the peripheral lymphoid organs of female mice, higher proportions of NKT cells were seen only in the spleen (0.57±0.05 % vs 0.25±0.02 %; p<0.001; Man-Whitney U test; Figure 4.17).

The increased susceptibility of diabetes in NOD.*Tnfsf10*$^{-/-}$ mice was not due to a defect in the numbers of immunoregulatory cells, as genetic ablation of TRAIL did not create a deficiency in neither $CD4^+CD25^+FoxP3^+$Tregs or αGal-Cer/CD1d-tetramer$^+$ NKT cells in NOD mice. Interestingly, NOD.*Tnfsf10*$^{-/-}$ mice had higher numbers of NKT cells in the thymus and periphery. In contrast, a flow cytometric enumeration of the numbers of NKT cells in the thymi and peripheral lymphoid organs of TRAIL deficient C57BL/6 mice revealed no difference when compared to the parental control strain (Figure 4.18).

4.2.6 Cytokine levels in NOD.*Tnfsf10*$^{-/-}$ mice post αGal-Cer treatment

NKT cells, like Tregs are capable of immediate regulatory function following TCR stimulation, however, unlike Tregs, NKT cells are not always inhibitory in action; they may also play a role in exacerbating autoimmunity (Fuss et al., 2004; Griseri et al., 2005; Jahng et al., 2001). This can be attributed to their

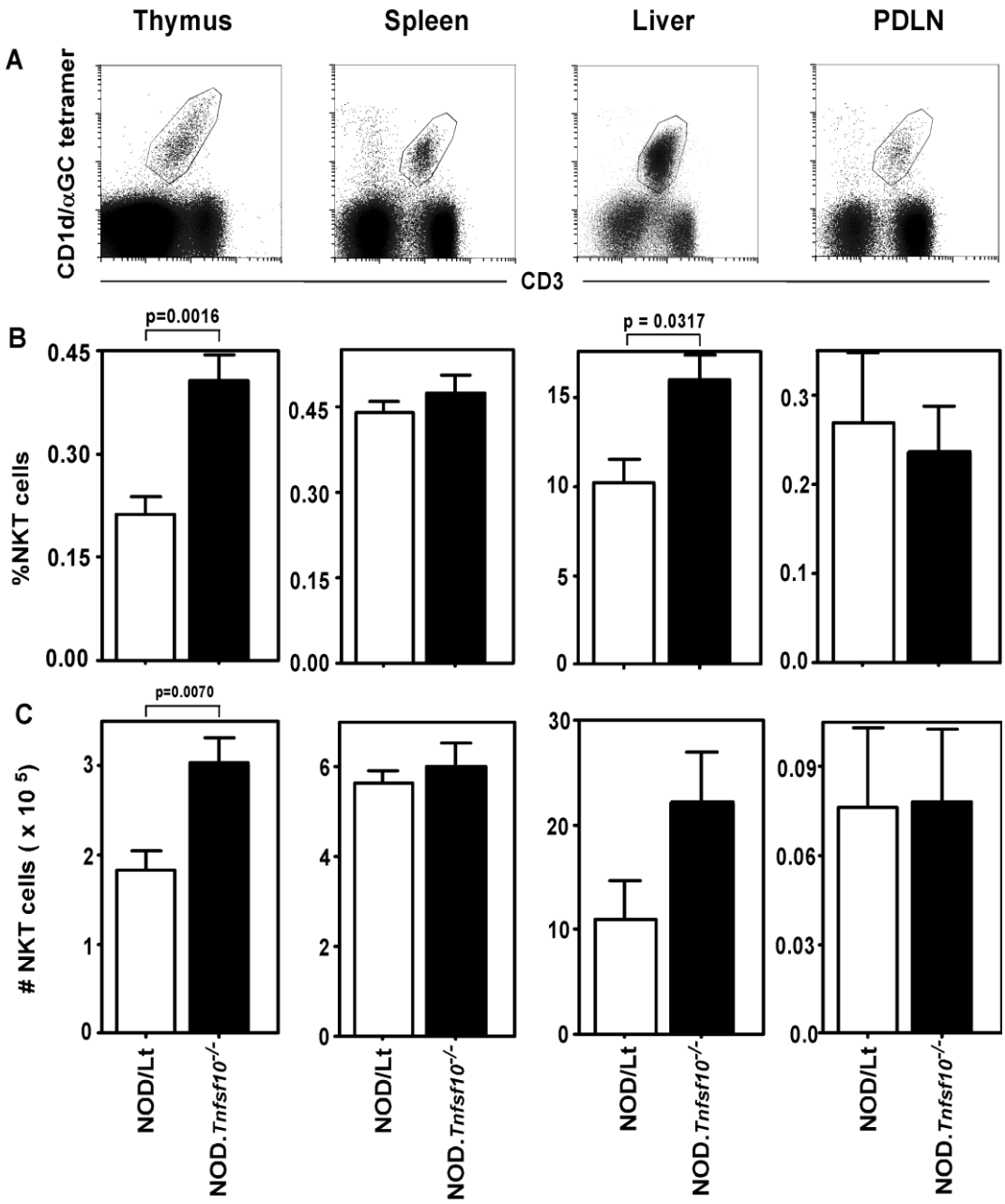


Figure 4.16 NKT cells in male NOD.*Tnfsf10*$^{-/-}$ mice. Representative plots of proportions of CD1d/ αGal-Cer tetramer-positive T cells (CD3 positive) in the thymi, spleens, livers and pancreatic draining lymph nodes (PDLN) of male NOD.*Tnfsf10*$^{-/-}$ (n=5) and NOD/Lt (n=5) mice (A). NKT cell proportions (mean ± SEM), absolute numbers (mean ± SEM) represented by histograms (B and C respectively). Significance by Mann-Whitney U test; values are not corrected for multiple hypothesis testing.

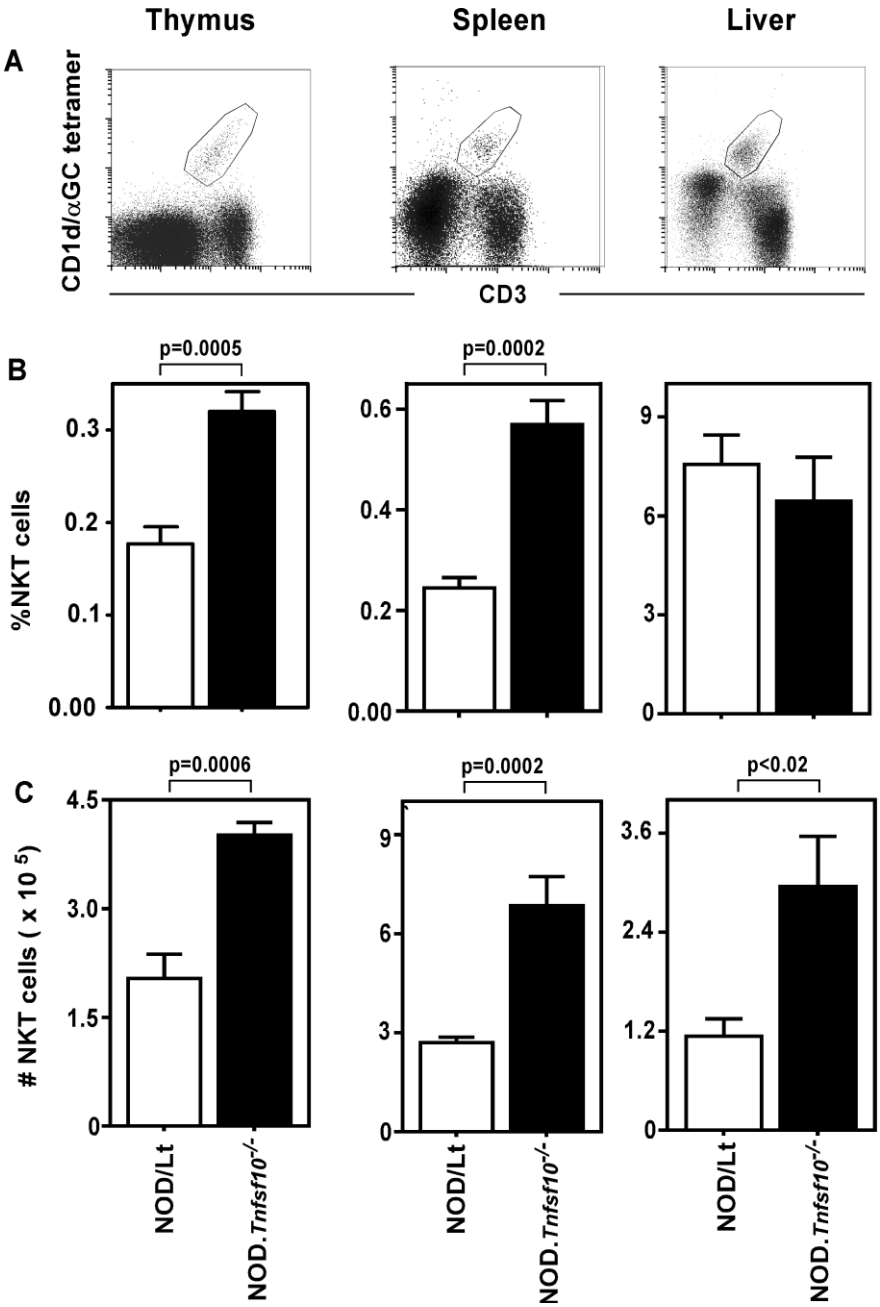


Figure 4.17 NKT cells in female NOD.*Tnfsf10*$^{-/-}$ mice. Representative plots of proportions of CD1d/ αGal-Cer tetramer-positive T cells (CD3 positive) in the thymi, spleens, livers and pancreatic draining lymph nodes (PDLN) of male NOD.*Tnfsf10*$^{-/-}$ (n=8) and NOD/Lt (n=8) mice (A). NKT cell proportions (mean ± SEM), absolute numbers (mean ± SEM) represented by histograms (B and C respectively). Significance by Mann-Whitney U test; values are not corrected for multiple hypothesis testing.

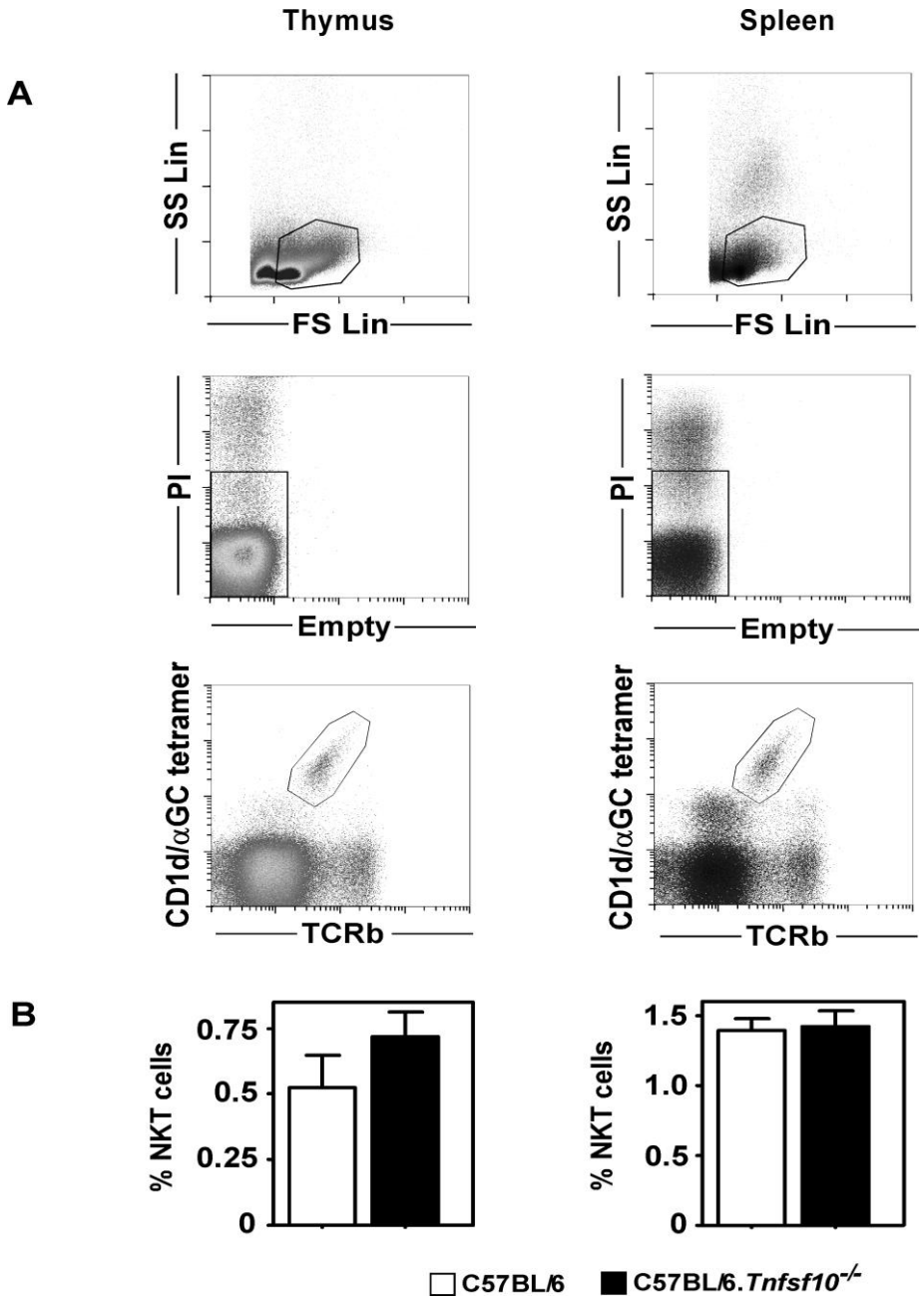


Figure 4.18 NKT cells in C57BL/6.*Tnfsf10*$^{-/-}$ mice. Proportions of the thymic and splenic NKT cells in female C57BL/6 and C57BL/6.*Tnfsf10*$^{-/-}$ mice. Thymocytes and splenocytes from C57BL/6 (n=5) and C57BL/6.*Tnfsf10*$^{-/-}$ (n=5) mice were analysed on a flow cytometer. Viable cells were gated while an empty vs PI plot was used to exclude dead and auto fluorescent cells (A). The percentages of CD1d/ GalCer tetramer-positive T cells (TCR positive) were compared between C57BL/6 and C57BL/6.*Tnfsf10*$^{-/-}$ mice and represented in the histograms (B).

ability to promptly produce proinflammatory cytokines. Therefore, it is possible that an elevation in NKT cell numbers and function could play a proinflammatory role and exacerbate disease in models of autoimmunity. To determine whether the increased susceptibility of diabetes in NOD.*Tnfsf10*$^{-/-}$ mice was due to the increased NKT cell numbers, functional analysis was performed by *in-vivo* activation of NKT cells with 2μg of αGal-Cer injected i.v.. The quantitative analysis of the secreted cytokines using cytometric bead array revealed no significant functional difference in NKT cell activity between TRAIL deficient NOD.*Tnfsf10*$^{-/-}$ mice and parental NOD/Lt strain (Figure 4.19), suggesting that the elevated numbers of NKT cells in NOD.*Tnfsf10*$^{-/-}$ mice played no role in its increased susceptibility to diabetes.

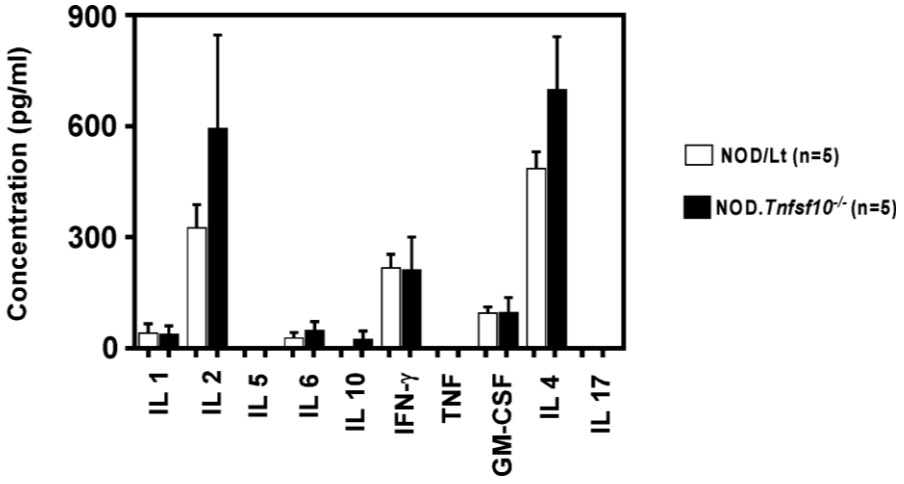


Figure 4.19 Cytokine levels in NOD.*Tnfsf10*$^{-/-}$ mice. No difference was observed between cytokine levels in NOD/Lt and NOD.*Tnfsf10*$^{-/-}$ sera measured two hours post 2mg α-GalCer treatment. Significance by Mann-Whitney U test. Error bars denote standard error of the mean.

4.2.7 Severity of insulitis in male NOD.*Tnfsf10*$^{-/-}$ mice

Histological analysis of the pancreata of pre-diabetic (10/52wk) TRAIL deficient male NOD mice revealed a dramatic increase in lymphocyte recruitment to the islets, compared to the wild type NOD/Lt mice (Figure 4.20, Table 4.1). While the pancreatic islets of NOD mice (306 islets scored) were normal or mildly infiltrated by leukocytes, severe insulitis and extensive destruction were observed in majority of islets of the mutant mice (385 islets scored). Similarly, more islets with high degree of infiltration (scale of 4) were observed in female NOD.*Tnfsf10*$^{-/-}$ mice (50 islets scored) compared to parental NOD/Lt mice (88 islets scored, Table 4.1). These findings suggested that the death receptor ligand TRAIL played a role in inhibiting islet infiltration in NOD mice.

Scale of insulitis	0	1	2	3	4
NOD/Lt	71	15	8	5	2
NOD.*Tnfsf10*$^{-/-}$	21	27	21	14	17
P	0.0001	ns	0.02	0.05	0.0001
NOD/Lt	30	36	21	8	5
NOD.*Tnfsf10*$^{-/-}$	39	25	11	4	21
P	ns	ns	ns	ns	0.0012

Table 4.1 The proportion of islets exhibiting various degrees of insulitis expressed as a percentage of the total number of islets scored. Insulitis scores for male and female NOD/Lt and NOD.*Tnfsf10*$^{-/-}$ mice are depicted on the upper and lower panels respectively. Islets of H&E stained pancreata were scored for the degree of insulitis on a scale of 0-5: 0, no infiltrate; 1, periislet infiltrate (<25%); 2, 25-50% accumulation of inflammatory cells; 3, 50-75% intraislet infiltration; 4, severe structural derangement and complete loss of cells. Comparison of NOD/Lt and NOD.*Tnfsf10*$^{-/-}$ insulitis scores was performed by Fisher's exact test.

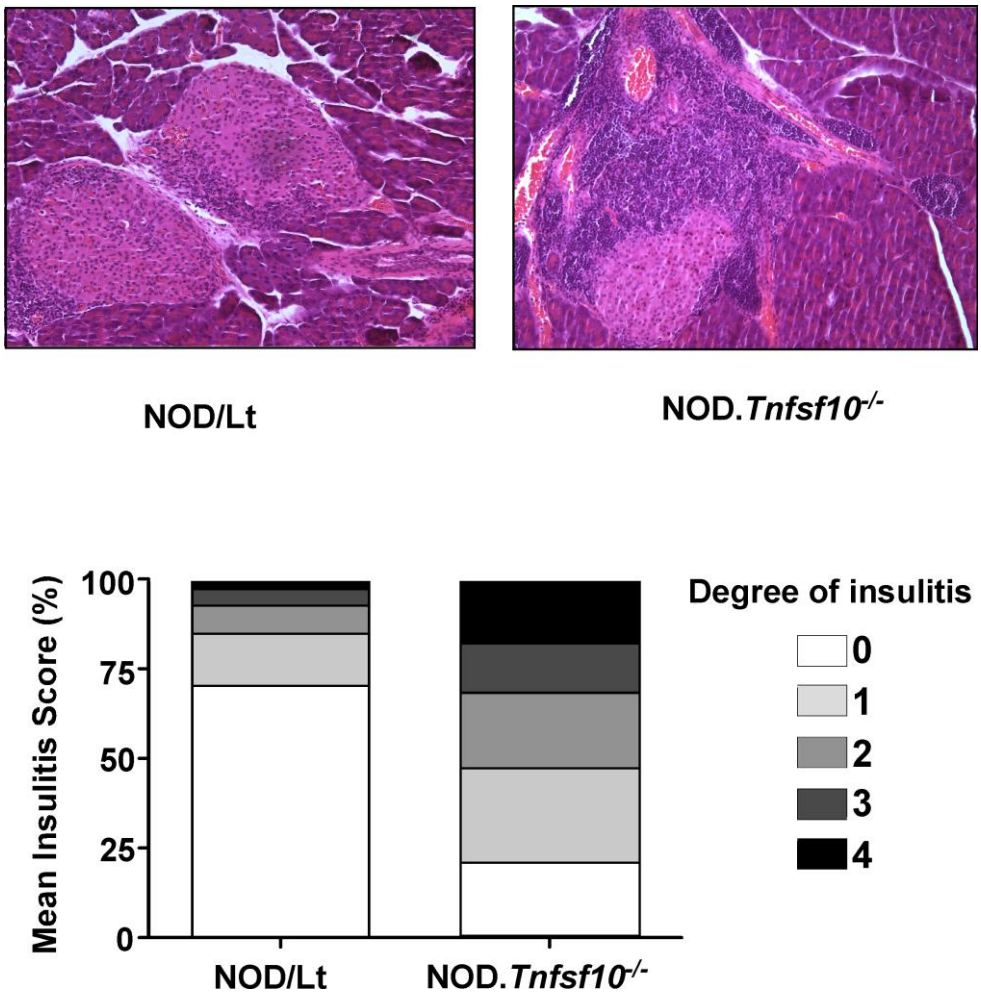


Figure 4.20 Histological analysis of H&E stained pancreas of NOD.*Tnfsf10*$^{-/-}$ mice. A total of 385 and 306 islets of age matched (10 week) male NOD.*Tnfsf10*$^{-/-}$ (n=10) and NOD/Lt (n=11) mice respectively, were scored for the degree of insulitis. Insulitis severity of H&E stained pancreata was scored on a scale of 0-4: 0, no infiltrate; 1, periislet infiltrate (<25%); 2, 25-50% accumulation of inflammatory cells; 3, 50-75% intraislet infiltration; 4, severe structural derangement and complete loss of cells. NOD.*Tnfsf10*$^{-/-}$ mice had more islets with higher degrees of insulitis (scales 2 to 4), while the parental NOD/Lt mice had more islets with modest levels of insulitis. Results of Fisher's exact analysis listed in Table 4.1

4.2.8 TRAIL expression on murine pancreatic islets

The increased severity of insulitis seen in 10-week old male NOD.*Tnfsf10*$^{-/-}$ mice is not consistent with a beta cell intrinsic protective effect but may be consistent with difference in local selectin/chemokine/integrin/cytokine expression. TRAIL signalling may therefore be a mechanism by which islet cells (including β-cells) inhibit infiltration and destruction. Such a mechanism would seem to require the expression of TRAIL on islets. Although BioGPS, the gene annotation portal of the Novartis Research Foundation (http://biogps.gnf.org/), reports no message for death receptors 4, 5 or decoy receptor 1 on human islets, TRAIL does appear to be expressed. In order to confirm TRAIL expression on murine β cells, islets isolated from young (7-8 weeks) male NOD/Lt mice and from NOD mice bearing the targeted deletional mutation of *Tnfsf10* were analysed by flowcytometry. While TRAIL expression was detected on wild type β cells, the targeted deletion of TRAIL in NOD mice eliminated this expression (Figure 4.21).

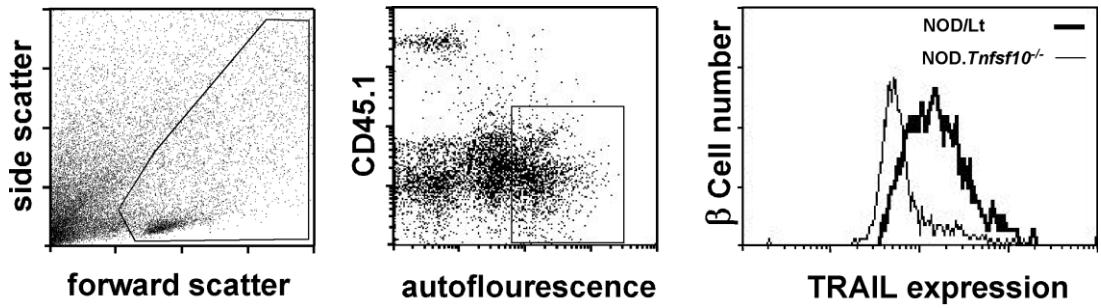


Figure 4.21 TRAIL expression on β-cells of NOD mice. Representative flowcytometry plot illustrating the elimination of TRAIL expression on NOD beta cells bearing targeted deletional mutation of *Tnfsf10*.

4.2.9 NOD.*Tnfsf10*$^{-/-}$ mice exhibit enhanced IP-10 secretion on LPS challenge

Chemokines are key signalling molecules that attract immune cells to the site of infection or inflammation. Interferon-γ inducible protein – 10kD (IP-10)/ CXCL10, the ligand of the CXCR3 chemokine receptor, is a member of the CXC subfamily of chemokines, which plays a crucial role in immune system development and deployment. This chemoattractant cytokine is mainly expressed by activated T cells, macrophages, endothelial cells, fibroblasts and keratinocytes in response to IFN-γ or TNF–α (Gattass et al., 1994; Luster and Ravetch, 1987). Higher levels of serum IP-10 levels in NOD mice are reported in the diabetic period (20-24 weeks of age) than in the pre-diabetic period (4,8 and 16 weeks of age) (Shigihara et al., 2006). TRAIL signalling has been reported to suppress the secretion of IP-10 (Secchiero et al., 2005). To test whether TRAIL signalling suppressed the levels of serum IP-10 in NOD mice, this chemokine was quantified in serum samples from NOD/Lt and NOD.*Tnfsf10*$^{-/-}$ mice using the FlowCytomix simplex bead assay. No difference in IP-10 levels was observed in the serum of 8 week-old (pre-diabetic) or 20 week-old NOD/Lt mice and NOD mice bearing the targeted deletional mutation of *Tnfsf10* (Figure 4.22A), suggesting that any differences in TRAIL signalling mediated chemokine suppression is more likely to be local rather than systemic. Recent experiments using RIP-LCMV mice suggest IP-10 expression on islet β-cells, in response to virus infection of the pancreas (Frigerio et al., 2002; Rhode et al., 2005). Further, *in vitro* stimulation of human

islets and rat β-cells by IFN-γ or IL-1β induced islet IP-10 secretion (Cardozo et al., 2003). Inorder to test the possibility that TRAIL signalling in pancreatic islets inhibits the secretion of chemokines responsible for T cell recruitment to the islets, IP-10 levels in the culture supernatants of islets from young (6-7 weeks) NOD.*Tnfsf10*$^{-/-}$ mice (4 replicates) and NOD/Lt mice (10 replicates) were determined. Islets isolated from either strain, stimulated in vitro with 1μg/ml LPS for 48 hours, secreted large amount of IP-10; however, the targeted deletional mutation of *Tnfsf10* appeared to significantly increase in IP-10 secretion in NOD mice (Figure 4.22B, $p<0.03$, Mann-Whitney test).

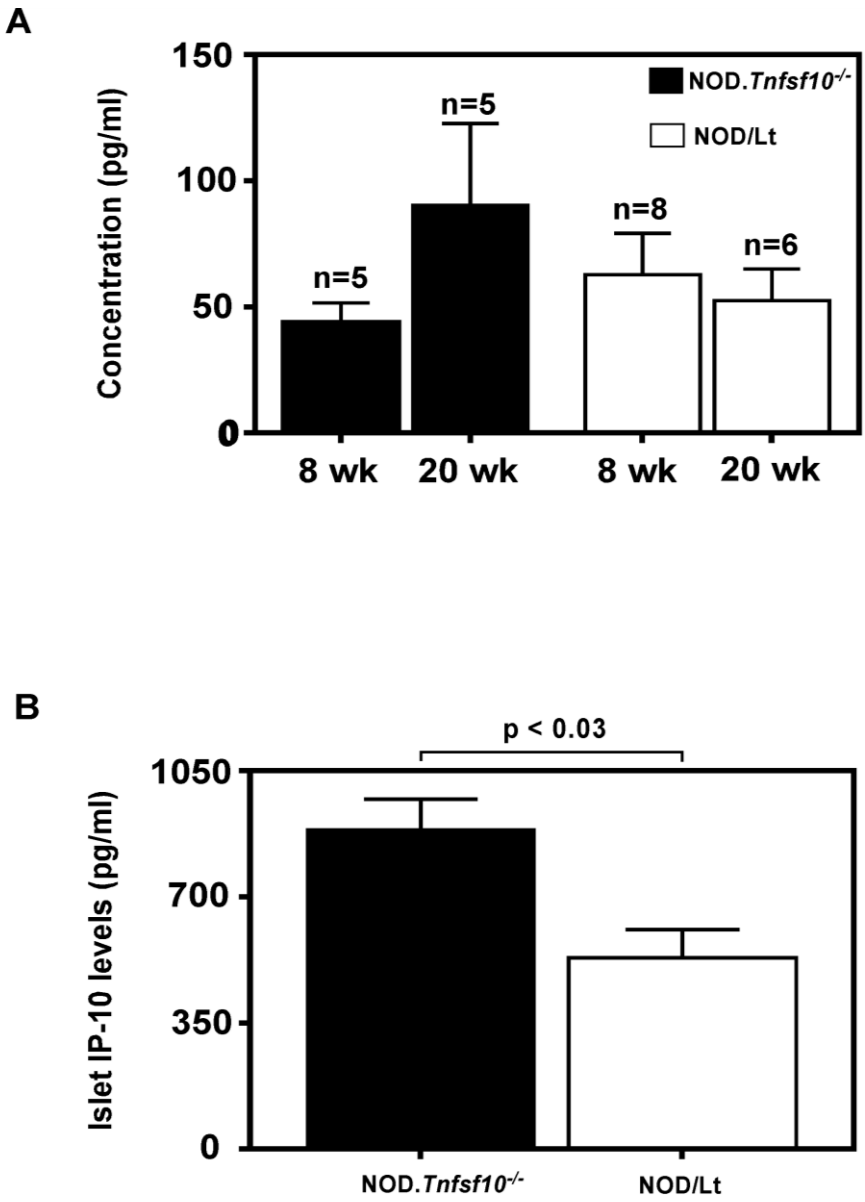


Figure 4.22 TRAIL mediated suppression of islet IP-10/CXCL10 secretion in NOD mice. Serum levels of IP-10 at 8 wks. and 20 wks. age in NOD.*Tnfsf10*$^{-/-}$ mice and NOD/Lt mice (A). IP-10 secretion by β-cells of TRAIL deficient NOD.*Tnfsf10*$^{-/-}$ mice (4 replicates) compared with secretion by β-cells from NOD/Lt mice (10 replicates) after 1mg/ml LPS challenge (B). Significance by Mann-Whitney U test.

4.3 Discussion

TRAIL, which is primarily expressed on the surface of activated NK cell, T cells, macrophages and dendritic cells, mediates immune surveillance and anti-tumor cytotoxicity (Bouralexis et al., 2005; Kayagaki et al., 1999; Smyth et al., 2003; Strebel et al., 2002; Takeda et al., 2002). TRAIL-bearing circulating immune cells selectively eliminate tumor cells, while constitutively protecting normal tissues (Kavurma et al., 2008; Klefstrom et al., 2002; Nesterov et al., 2004; Secchiero et al., 2004; Wang et al., 2005b). Unlike other members of TNF family, significant levels of TRAIL transcripts have been detected in many human tissues and cell lines (Bouralexis et al., 2005), suggesting additional roles. Contradictory results on the requirement of TRAIL in thymic negative selection as well as on its role in the suppression of T cell mediated autoimmunity exists in the literature, necessitating the employment of a robust approach to dissect the role of TRAIL at the molecular and cellular level. To achieve this, in collaboration with Thomas Kay and Mark Smyth, a targeted deletional mutation of *Tnfsf10* was backcrossed from C57BL/6 mice onto the NOD/Lt background. Genetic homogeneity of these mice was confirmed by typing 647 single nucleotide polymorphisms (Australian Genome Resource Facility, Melbourne, Australia).

Male NOD mice bearing targeted deletion of TRAIL (NOD.*Tnfsf10*$^{-/-}$), housed under tightly controlled environmental conditions at the SPF facility of Comparative

Genomincs Centre of James Cook University, trebled their incidence of spontaneous diabetes. TRAIL deficiency induced an earlier onset of diabetes in female NOD mice, but disease incidences in female mice were not significantly different. It was possible that the naturally high penetrance of spontaneous diabetes in female mice masked any evidence of an enhancement of this trait in a TRAIL deficient environment. However, the effects of targeted deletion of TRAIL on diabetes incidence are clearly seen in female NOD mice treated with cyclophosphamide (Figure 4.2B) or by adoptive transfer (Figure 4.11C). TRAIL blockade in NOD mice using a soluble TRAIL receptor (sDR5) exacerbated the onset of streptozotocin induced diabetes (Lamhamedi-Cherradi et al., 2003a) and enhanced adoptive transfer of the disease into NOD.*scid* mice by splenocytes from spontaneously diabetic NOD mice (Mi et al., 2003). Even though protein mediated blockade of TRAIL in the above studies suggested a potential role for TRAIL in NOD diabetes, our finding is the first and most robust demonstration of increased incidence of spontaneous diabetes in NOD mice bearing a targeted deletion of TRAIL. TRAIL deficiency did not affect BCG mediated protection of diabetes in NOD mice, suggesting that the actions of *M.bovis* and TRAIL are independent.

Previous reports have implicated TRAIL in thymic deletion (Corazza et al., 2004; Lamhamedi-Cherradi et al., 2003b). Such a role for TRAIL would provide an explanation for the increased severity of spontaneous diabetes in male NOD.*Tnfsf10*$^{-/-}$ mice. NOD mice express Mtv-3 and 9, which induce negative

selection of Vβ3$^+$ and Vβ5$^+$ reactive thymocytes (Brenden and Bohme, 1999; Fairchild et al., 1991; Tomonari et al., 1993). No difference in numbers or proportions of Vβ3$^+$ or Vβ5$^+$ T cells between male NOD.*Tnfsf10*$^{-/-}$ and parental NOD/Lt mice was found, suggesting a limited role for TRAIL in in the thymic selection process. This finding is in agreement with normal negative selection reported in TRAIL deficient B6 mice (Cretney et al., 2003) and also in DR5 deficient mice (Diehl et al., 2004). Moreover, thymic negative selection was found to be unaffected in mice that had impaired TRAIL induced cytotoxicity due to transgenic expression of CrmA or a dominant negative mutant FADD protein (Walsh et al., 1998). It was therefore unlikely that differences in thymic deletion were responsible for the differences observed in diabetes incidence.

Besides thymic deletion, TRAIL also plays a role in the induction of peripheral tolerance. In a recent study, adoptive transfer of TRAIL-overexpressing murine T cells was reported to induce apoptosis of alloreactive T cells, in a DR5-dependent manner (Ghosh et al., 2013). Griffith and colleagues developed a tolerance model in which TRAIL-mediated-apoptosis inhibited the expansion of islet-antigen-reactive CD4$^+$ T cells (Griffith et al., 2007). Previous studies have demonstrated functional TRAIL-R expression on activated T cells in human and mouse, but not on resting T cells (Herbeuval et al., 2005; Zhang et al., 2003), suggesting TRAIL mediated regulation of T cell activity. Such a role for TRAIL could also account for the increased severity of spontaneous diabetes found in NOD.*Tnfsf10*$^{-/-}$ mice.

However, there was no evidence of a systemic increase in numbers or proportions of activated and memory phenotype $CD4^+$ and $CD8^+$ T cells in the peripheral T cell compartments of NOD.*Tnfsf10*$^{-/-}$ mice compared to parental NOD/Lt mice. Furthermore, adoptive transfer of splenocytes from male or female mice of either strain into NOD.*scid* recipients did not reveal any significant difference in their diabetogenic activity. These findings indicated that TRAIL was unlikely to inhibit β-cell destruction by eliminating or suppressing the functions of autoreactive T cells in the periphery. The increased susceptibility of diabetes in NOD.*Tnfsf10*$^{-/-}$ mice was not due to a defect in the numbers of immunoregulatory cells, as genetic ablation of TRAIL did not create a deficiency in $CD4^+CD25^+FoxP3^+$ Tregs or αGal-Cer/CD1d-tetramer$^+$ NKT cells in central or peripheral lymphoid organs of NOD mice. However slightly increased NKT cell proportions were observed in the thymi and spleens of NOD.*Tnfsf10*$^{-/-}$ mice, but these did not appear to be of functional significance (Figures 4.16 and 4.17). Interestingly, reciprocal spleen cell transfers into irradiated recipients performed between NOD.*Tnfsf10*$^{-/-}$ and NOD/Lt mice revealed that susceptibility to adoptive transfer of diabetes depended on the genotype of the recipient, not donor. This suggested a protective effect of TRAIL signalling on nonhematopoietic tissues. However, the resistance of NOD.*Tnfsf10*$^{-/-}$ mice to diabetes induced by a single dose SZ treatment (a β-cell toxin) is not consistent with TRAIL playing an intrinsic role in β-cell protection.

Even as the mechanism(s) or cells responsible for the diabetes-protective effect of TRAIL remain unclear, its ability to inhibit lymphocytic infiltration and

inflammation has been previously demonstrated. In EAE, an induced model of multiple sclerosis (Baxter, 2007), TRAIL blockade by anti-TRAIL monoclonal antibody, as well ablation of TRAIL by targeted gene deletion, led to a higher degree of inflammation in the central nervous system, and a more severe disease (Cretney et al., 2005; Hilliard et al., 2001). In experimental autoimmune thyroiditis, recombinant TRAIL treatment caused a significant decrease in mononuclear infiltration in the thyroid, resulting in less thyroid follicular destruction (Wang et al., 2005a). Consistent with TRAIL's ability to inhibit lymphocytic infiltration, NOD.*Tnfsf10*$^{-/-}$ mice showed a dramatic increase in lymphocyte recruitment to the pancreatic islets. The increased severity of insulitis and extensive destruction observed in majority of islets of 10-week old male NOD.*Tnfsf10*$^{-/-}$ mice was not consistent with a β-cell intrinsic protective effect, but the rampant insulitis observed in TRAIL-deficient NOD mice was similar to the phenotypes of mice bearing transgenes for pro-inflammatory cytokines expressed under the rat insulin promoter, suggesting that the mutant mice over expressed one or more cytokines or chemokines. Freshly isolated NOD islets secreted significant levels of chemokines, especially IP-10, but the islets from diabetes-resistant NOR mice (characterised by negligible insulitis) and from BALB/c mice, secreted only negligible levels of chemoattractants (Giarratana et al., 2004), suggesting constitutive secretion of chemokines to be a possible contributing factor in β-cell destruction. IP-10 is a chemokine that specifically attracts CXCR3-expressing effector CD8 T cells, on which the pathogenesis of diabetes in NOD mice

is dependent. This chemoattractant cytokine is implicated in human T1D, as elevated serum levels of chemokines including IP-10 have been reported in diabetes patients and in subjects at risk of developing the disease (Lohmann et al., 2002; Nicoletti et al., 2002; Schloot et al., 2002; Shimada et al., 2001). IP-10 neutralisation by monoclonal antibody treatment halved cyclophosphamide precipitated diabetes in NOD mice and CXCR3-deficient transgenic mice had a delayed onset of the disease (Frigerio et al., 2002; Morimoto et al., 2004). Islet-specific expression of IP-10 in RIP-NP-CXCL10 transgenic mice accelerated the autoimmune process by enhancing the migration of antigen-specific lymphocytes to their target site (Rhode et al., 2005). All these findings suggest IP-10 to be a chemoattractant of islet-specific effector cells. Secchiero and colleagues demonstrated that exposure of endothelium to TRAIL caused a significant reduction in leukocyte adhesion and inhibition of secretion of IP10 and MCP2 (Secchiero et al., 2005). The dramatic increase in lymphocyte recruitment to the pancreatic islets seen in NOD.*Tnfsf10*$^{-/-}$ mice is consistent with a role for TRAIL in inhibiting islet chemokine secretion. In accordance with the above hypothesis, e*x vivo* IP-10 secretion by islets from NOD.*Tnfsf10*$^{-/-}$ mice was found to be significantly higher than the chemokine secreted by islets from parental NOD/Lt mice.

The result of the islet-chemokine assay is consistent with constitutive TRAIL signalling inhibiting the release of chemoattractants in pancreatic islets. However, it is still unclear how TRAIL signalling inhibits islet chemokine secretion. IFN-γ is

known to induce pancreatic islet-cells to secrete several chemokines including IP-10 (Cardozo et al., 2001a; Cardozo et al., 2001b; Cardozo et al., 2003). Previous studies demonstrated that IFN-γ–secreting T cells were sensitive to TRAIL-induced apoptosis (Roberts et al., 2003; Zhang et al., 2003) and that TRAIL-R-deficient mice had elevated levels of IFN-γ (Diehl et al., 2004), suggesting a suppressive role for TRAIL signalling on IFN-γ production. In a mouse model of autoimmune thyroiditis, the presence of TRAIL was reported to be associated with IFN-γ downregulation, which in turn regulated chemokine production, thereby reducing lymphocytic infiltration into thyroid glands (Wang et al., 2005a). Considering the above findings, it appears that IFN-γ-induced-chemokines are involved in the destruction of pancreatic islet cells. Moreover, there exists the possibility of a "dialogue" between invading lymphocytes and the target islet-cells during the course of insulitis (Figure 4.23A). Thus, activated effector cells homing into the islets, produce proinflammatory cytokines, including IFN-γ, inducing β-cells to release chemokines including IP-10. These chemokines are likely to create a chemokine gradient that will attract additional mononuclear cells. Uninterrupted, this vicious cycle, will exacerbate islet infiltration and β-cell destruction. In view of the observations made in this study, I hypothesize that TRAIL on islets, by inhibiting IFN-γ, as well as IFN-γ-induced-chemokines from T cells, checks this cycle of β cell destruction (Figure 4.23 B). In a recent study, adenoviral mediated systemic delivery of TRAIL in NOD mice conferred protection against diabetes by activating tissue inhibitor of metalloproteinase 1 (TIMP-1),

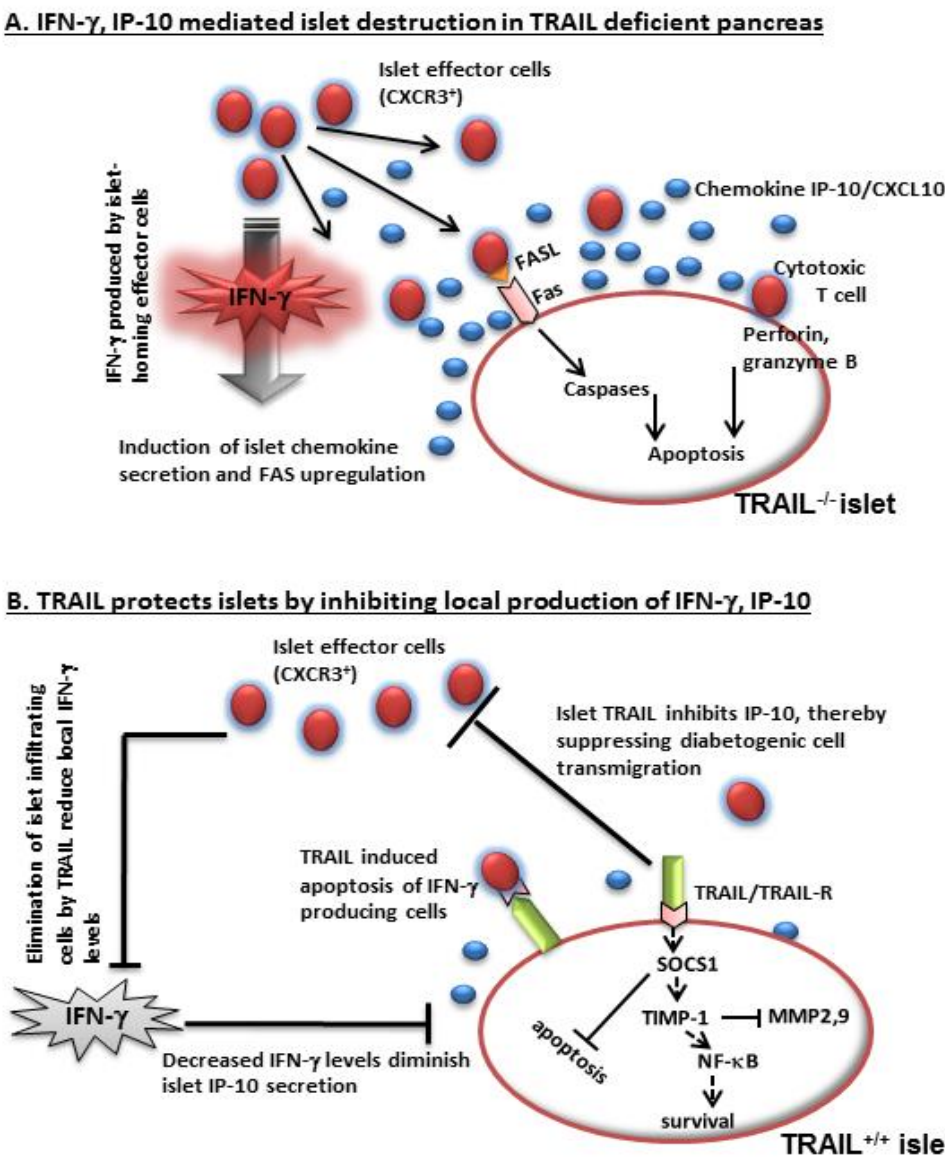


Figure 4.23 Proposed model of TRAIL mediated islet-cell protection. In a TRAIL deficient environment (NOD.*Tnfsf10*$^{-/-}$ mice) activated effector cells homing into the islets, produce proinflammatory cytokines, including IFN-γ, inducing β-cells to release chemoattractants including IP-10, thereby creating a chemokine gradient that attracts additional mononuclear cells (A). TRAIL interrupts this amplification feedback loop by inhibiting islet IP-10 secretion and by eliminating islet-infiltrating cells that produce IFN-γ (B). Moreover, TRAIL signalling confers protection to pancreatic β-cells by elevating levels of SOCS1, TIMP-1 or via the NF-κB survival response (B).

a natural inhibitor of matrix metalloproteinase (MMP 2 and 9) activity (Kang et al., 2010). TIMP-1 upregulation protected pancreatic β cells from cytokine-induced apoptosis as well as suppressed the transmigration of diabetogenic T cells into pancreatic islets, possibly since MMP-mediated proteolysis increases chemotactic potency of certain chemokines (Van den Steen et al., 2000; Van Den Steen et al., 2003). Suppressor of cytokine secretion 1 (SOCS1), a protein known to moderate the sensitivity of β-cells to key inflammatory cytokines associated with diabetes (Chong et al., 2002; Eizirik et al., 2009), was found to be elevated in human pancreatic islets in response to *in vitro* recombinant TRAIL exposure. Similarly, TRAIL-treated NOD mice had reduced severity of diabetes, and this was associated with increased pancreatic SOCS1 mRNA (Zauli et al., 2010). These recent findings suggest additional TRAIL signalling pathway mediated mechanisms of β cell protection (Figure 4.23 B).

In summary, we have found that targeted deletion of TRAIL in male autoimmune diabetes-prone NOD mice trebled the incidence of diabetes in this line. Paradoxically, this effect did not appear to affect the generation or regulation of diabetogenic T cells and the trait was not haematopoietic cell intrinsic in adoptive transfers. In contrast, it was associated with a dramatic increase in lymphocyte recruitment to the pancreatic islets that overexpressed chemoattractants, consistent with a previously reported, inhibitory effect of TRAIL signalling on chemokine production.

.....

CHAPTER 5

.....

POLYMORPHISM IN A FAS LIGAND SIMPLE SEQUENCE REPEAT AFFECTS TYPE 1 DIABETES IN NOD MICE

6.1 Introduction

The FAS ligand (Apo-1 Ligand/CD95 Ligand/*Tnfsf6*), a 40-kDa type II transmembrane protein of the TNF superfamily, is a key apoptotic signalling entity in the extrinsic apoptotic pathway. Defective FAS death receptor and/or its ligand are associated with several pathological conditions in both mouse and man including autoimmune disorders, lymphoproliferative syndrome and cancer (Adachi et al., 1996; Del-Rey et al., 2006; Fisher et al., 1995; Hashemi et al., 2013; Krammer, 2000; Peter et al., 2005; Rieux-Laucat et al., 1995; Sneller et al., 1992; Sun et al., 2004; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992; Wu et al., 2003; Zhang et al., 2005), suggesting a crucial role for FAS ligand in the maintenance of immune tolerance.

Transcription levels of *Fasl* are affected by allelic variations of its coding or promoter sequences. A single-nucleotide polymorphism in CAAT/enhancer-binding protein β (C/EBPβ) element in the enhancer region of human *Fasl* affects basal gene expression both *in vitro* and *in vivo*. The higher activity allele of the *Fasl* C/EBPβ element contributes to the development of systemic autoimmunity (Wu et al., 2003). Two naturally occurring alleles of *Fasl* in mice, *mFasL.1* (B6, C3H, MRL, SJL, NOD, NZB and NZW) and *mFasL.2* (BALB/c, DBA/1 and DBA/2) differ functionally (Kayagaki et al., 1997), FASL in the latter allotype is more effective in lysing FAS-bearing target cells. These findings suggest that

genetic polymorphism/s of CD95 ligand or its promoter sequence affect *Fasl* expression, and that FASL function depends on the context and gene expression levels.

Fasl gene expression is controlled by a host of transcription factors, including, NFAT (Latinis et al., 1997), NF-κB (Matsui et al., 1998), AP-1 (Kasibhatla et al., 1998), Sp1 (Kavurma et al., 2001) etc. The GATA transcription factors (GATA-1 to GATA-6), having conserved C_4 zinc finger DNA-binding domains, act as *cis*-regulatory elements in the expression of diverse genes involved in differentiation and proliferation in a wide range of cells and tissues (Orkin, 1992). GATA-1, GATA-2 and GATA-3 are mainly expressed in the hematopoietic system (Weiss and Orkin, 1995). GATA-4, GATA-5 and GATA-6 are expressed in several tissues, including intestine, lung and the heart (Molkentin, 2000). Previous studies indicate that GATA transcription factors can activate (Chin et al., 1995; Fung et al., 2005; Kieffer et al., 2002; Ko et al., 1991; Lee et al., 2001; Masuda et al., 2004; Migliaccio et al., 1993) , or repress (Chen et al., 2001; Imagawa et al., 1997; Kaminuma et al., 2004; Ramchandran et al., 2000; Schwenger et al., 2005; Usui et al., 2003), the expression of various genes, depending on the cellular context (Wei et al., 2011). Deletion of *Gata3*, affected the expression of 623 genes in mouse T cells, including the upregulation of *Fasl* (Wei et al., 2011), suggesting an inhibitory role for GATA-3 on *Fasl* expression.

Simple sequence repeats (SSR), or microsatellites, are polymorphic genetic loci with short tandem polynucleotide repeats. Such repetitions occur primarily due to slipped-strand mispairing, a unique mutational process, and subsequent error(s) during DNA replication, repair, or recombination (Levinson and Gutman, 1987). Mutations occur in these loci by insertions or deletions of one or a few repeat units (Wierdl et al., 1997). One such locus, *D1Bax208*, located immediately 3' to *Fasl* in NOD/Lt strain, was found to be highly mutable. *D1Bax208* microsatellite consisted of tandem GATA repeats, which incorporated the consensus DNA sequence, WGATAR (in which W denotes A/T, and R denotes A/G), the binding sites for GATA transcription factor family. It is therefore possible that, if GATA transcription factors bind to GATA elements in *D1Bax208* lying immediately 3' to *Fasl,* polymorphism at this microsatellite could modify the level of transcription in a dose dependent manner.

This study utilises NOD mouse lines carrying 2 minor alleles of *D1Bax208* (NOD.*D1Bax208*^{m1} mice and NOD.*D1Bax208*^{m2} mice). Experiments were designed to test the hypothesis that the variation in the repeat length of *D1Bax208* (putative binding sites for transcription factors GATA1 and GATA3), adjacent to *Fasl* in NOD mouse lines could cause allelic variation of *Fasl e*xpression and alter the diabetes incidence in these mice.

6.2 Results

5.2.1 Genotyping the NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice

A project performed in our laboratory aimed at analysing the genetic control of NKT cell numbers in NOD mice required large-scale genome screening. Over 300 established and novel tandem repeat sequences were characterised and primer pairs produced for this purpose. While screening the genome of an inbred NOD line, a member of the laboratory (Tatiana Toutsman), incidentally observed the expression of different alleles in the NOD background in one of the tandem repeats, *D1Bax208,* which lies between and immediately 3' to both *Fasl* and a putative glutamic pyruvate transaminase (alanine aminotransferase) 2 gene *Gm5049* (Figure 5.1). Female NOD/Lt mice were purchased from Jackson Laboratory (Maine, USA) and from ARC in Perth and analysed for polymorphism at *D1Bax208*, identifying 4 alleles in total. The most common allele was 179bp long, and the three minority alleles were 167bp, 187bp and 171bp. The 167bp allele was found only in a single lineage of mice obtained from ARC, while the 187bp allele was found in 9/50 NOD mice from Jackson laboratory and 8/92 NOD mice from ARC. The fourth allele (171bp) was found in 4/130 NOD mice from the ARC. This degree of variation is unlikely to be due to genetic contamination, because none of the 13 other polymorphic markers on chromosome 1, or any other marker of 137 tested on the rest of the autosomes showed any variability. It was therefore likely that the *D1Bax208* microsatellite is relatively highly mutable. The NOD line

carrying the 167bp allele was named NOD.*D1Bax208*^{m1}, while the line carrying the 179bp allele was named NOD.*D1Bax208*^{m2}. Mice expressing the 187bp allele and 171bp allele were not available for this study.

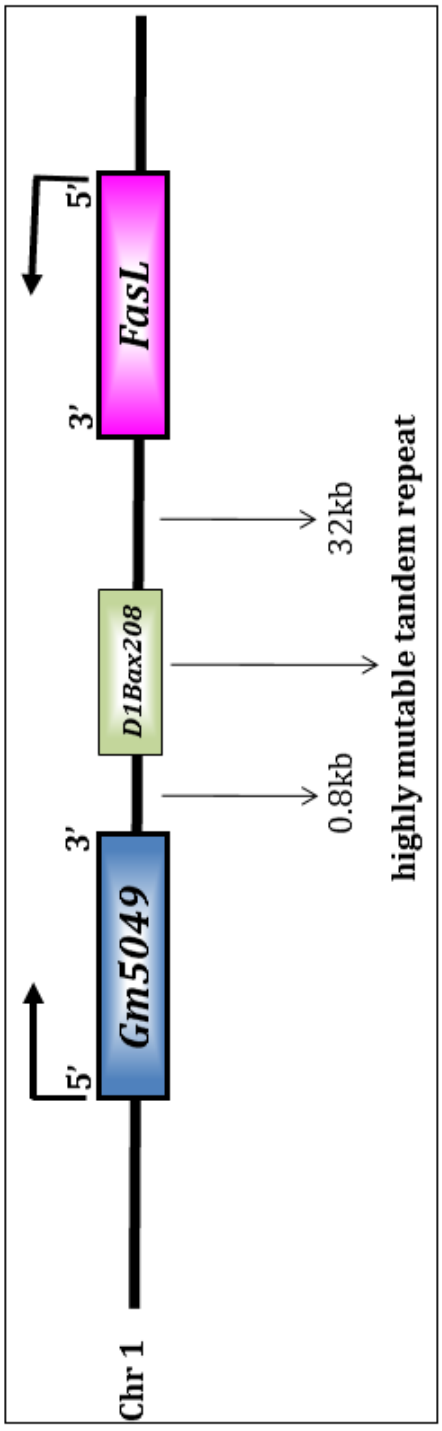


Figure 5.1 Genetic map of *D1Bax208*. Physical map indicating the genetic location of *D1Bax208*, depicting known genes that flank the highly mutable tandem repeat and the distance between them (kb).

The *D1Bax208* loci from control NOD/Lt mice and from a NOD congenic line with a C57BL/6 segment and from the NOD lines carrying the mutant alleles were amplified (Figure 5.2) and sequenced (Australian Genome Research Facility (AGRF), Melbourne, Australia; Table 5.1). Alignment of *D1Bax208* sequences from NOD.*D1Bax208*^{m1} mice (which lacked eight nucleotides compared to NOD/Lt) and NOD.*D1Bax208*^{m2} mice (which had extra four nucleotides compared to NOD/Lt) with that of the wild type NOD/Lt mice revealed a variation in number of putative transcription factor (GATA) binding sites among these mouse lines (Figure 5.3). Members of GATA transcription factor family are zinc-finger transcription factors that bind to the consensus DNA sequence, WGATAR (W = A/T; R = A/G). The analysis of *D1Bax208* sequence using Transcription Element Search System (TESS; http://www.cbil.upenn.edu/tess), a web tool for predicting transcription factor binding sites in DNA sequences, revealed putative binding sites for the transcription factors GATA-1, GATA-3 and GATA-4 within this locus (Figure 5.4). The variation in (GATA) repeat numbers at *D1Bax208* in NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice (a difference of 3 (GATA) repeats) resulted in over 16% difference in the number of putative GATA binding sites at this locus as summarised in Table 5.2.

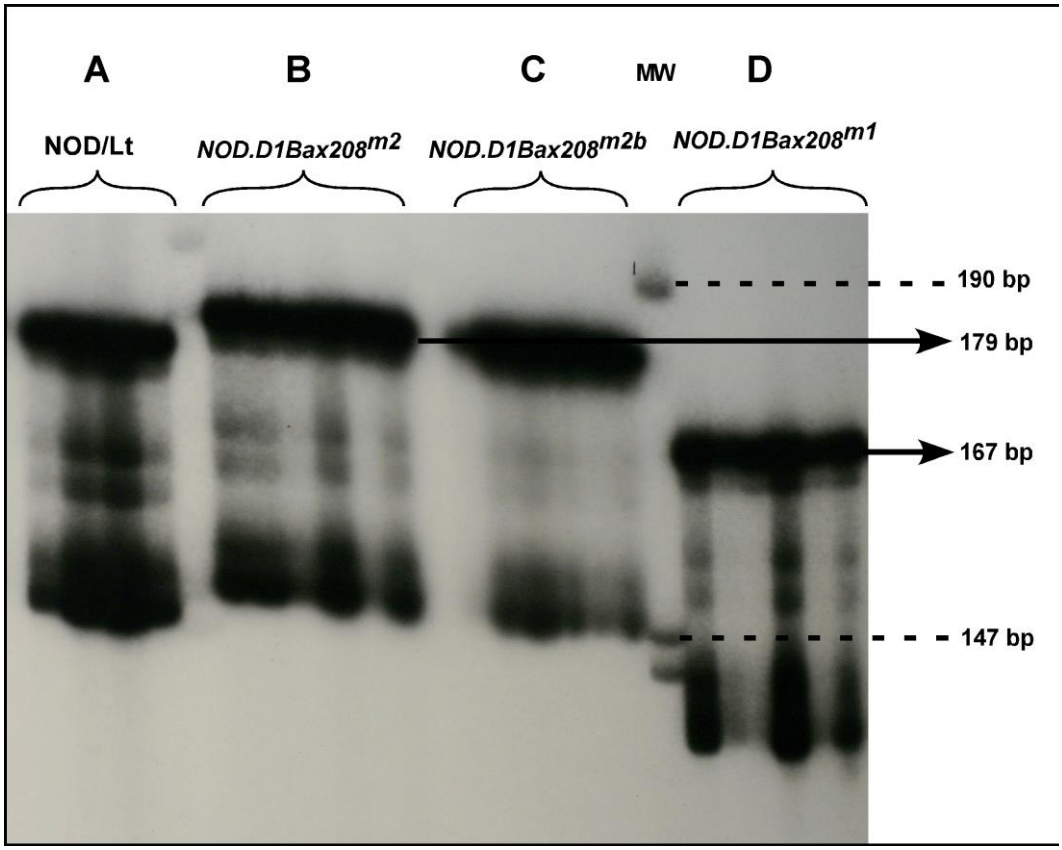


Figure 5.2 PCR amplification products of the *D1Bax20*8 tandem repeat resolved by polyacrylamide gel electrophoresis (PAGE). PAGE profile of the amplicons of *D1Bax208* locus demonstrating the wild type allele (A, 175bp, NOD/Lt) and the two mutant alleles identified in the inbred NOD line (B, 179bp, NOD.*D1Bax208*^{m2}) and (D, 167bp, NOD.*D1Bax208*^{m1}). Amplification profile of the *D1Bax208* microsatellite in NOD.*D1Bax208*^{m2b}, congenic with a C57BL/6 segment (C, 171bp). 'MW' indicates the lane with the molecular weight marker (190bp and 147bp).

NOD lines	*D1Bax208* tandem repeat	Size (bp)
NOD/Lt	**AGCGAATGGATCCATGAGTG**GATGGATGGGTATGGTAGATGGATAGATAGATAGATAG ATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATGATAGATAGATAG ATGAGAGAGAGAGAGAGAGATGCTTCGATGGGTGGAT**GGATCTAAGCAACGTTGGTTG**	175
NOD.*D1Bax208*^{m1}	**AGCGAATGGATCCATGAGTG**GATGGATGGGTATGGTAGATGGATAGATAGATAGATAG ATAGATAGATAGATAGATAGATAGATAGATAGATAGATGATAGATAGATAGATGAGAGA GAGAGAGAGAGATGCTTCGATGGGT**GGATGGATCTAAGCAACGTTGGTTG**	167
NOD.*D1Bax208*^{m2}	**AGCGAATGGATCCATGAGTG**GATGGATGGGTATGGTAGATGGATAGATAGATAGATAG ATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATGATAGATAGATAG ATAGATGAGAGAGAGAGAGAGAGATGCTTCGATGGGTGGAT**GGATCTAAGCAACGTTG GTTG**	179
NOD.*D1Bax208*^{m2b}	**AGCGAATGGATCCATGAGTG**GATGGATGGGTATGGTAGATGGATAGATAGATAGATAG ATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGATGATAGATAGATAGATGA GAGAGAGAGAGAGAGATGCTTCGATGGGTGGAT**GGATCTAAGCAACGTTGGTTG**	171

Table 5.1 Sequence of *D1Bax208* alleles from NOD/Lt, NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2} and NOD.*D1Bax208*^{m2b}. Sequencing data of PCR amplified products of *D1Bax208* loci from control NOD/Lt mice and from the NOD lines carrying the mutant alleles, confirming the absence of any new polymorphism at this locus. The nucleotides indicated by bold letters represent the primer pairs used for *D1Bax208* amplification.

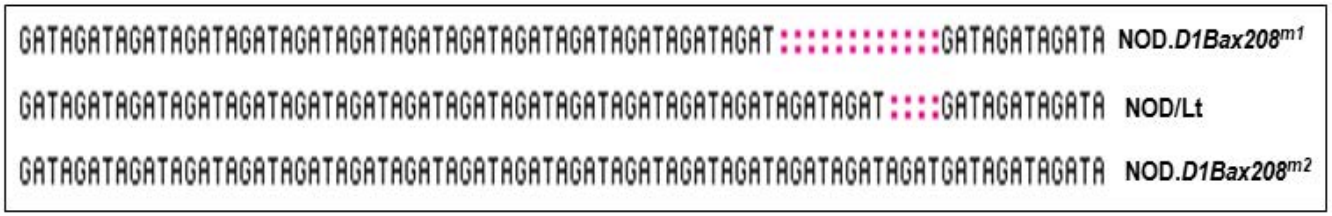


Figure 5.3 Alignment of DNA sequences from *D1Bax208* loci of NOD/Lt NOD.*D1Bax208*^{m1}, NOD/Lt and NOD.*D1Bax208*$^{m2.}$. Gap placements were done (A), using Sequencher (v 4.1, Gene Codes Corporation), for aligning the NOD.*D1Bax208*^{m1}, NOD/Lt and NOD.*D1Bax208*^{m2} DNA sequences.

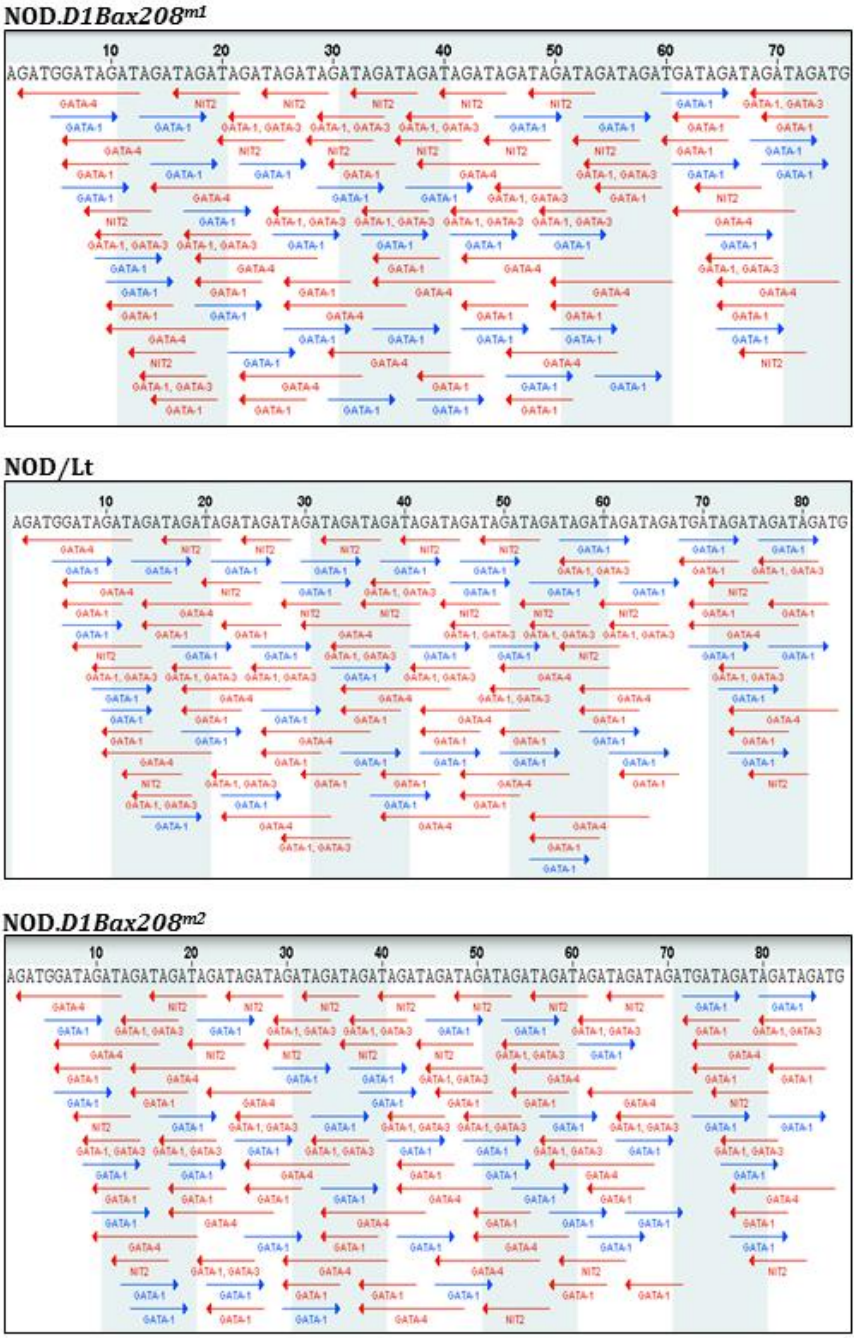


Figure 5.4 TESS output depicting the putative binding sites for transcription factors at *D1Bax208.* Analysis done using TESS (http://www.cbil.upenn.edu/tess), a web tool for predicting transcription factor binding sites in DNA sequences.

Transcription factor binding sites	NOD.*D1Bax208*^{m1}	NOD/Lt	NOD.*D1Bax208*^{m2}
GATA 1	**63**	**71**	**75**
GATA 3	**14**	**16**	**17**
GATA 4	**15**	**17**	**18**

Table 5.2 Variation in the numbers of GATA1, GATA3 and GATA4 transcription factor binding sites at *D1Bax208* in the three NOD lines. Analysis performed using TESS (http://www.cbil.upenn.edu/tess), a web tool for predicting transcription factor binding sites in DNA sequences.

5.2.2 FASL expression in NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice

Polymorphisms in *D1Bax208* affect putative GATA binding sites as identified in the sequence analysis (Table 5.2). Among the members of the GATA family of transcription factors, GATA-1, 2, and 3 are active in haemopoetic cells of vertebrates. Binding of these transcription factors to GATA elements in the human erythropoietin gene promoter negatively regulated its expression (Imagawa et al., 1997). Overexpression of GATA-3 inhibits *IL7Rα, M-CSF-R, PreTα, Rag-1 and Rag-2* expression, and upregulates *c-kit and SCL* in mouse T cells (Anderson et al., 2002; Taghon et al., 2007). GATA-4 is implicated in controlling various murine genes involved in cardioprotection, ovarian development and function and somatic cell reprogramming (Broderick et al., 2012; Laitinen et al., 2000; Serrano et al., 2013).

It is therefore possible that, if GATA transcription factors bind to GATA elements in *D1Bax208* lying immediately 3' to *FasL,* polymorphism at this microsatellite could modify the level of transcription in NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice in a dose dependent manner. Con A, a plant mitogen originally extracted from the jack bean *Canavalia ensiformis*, is known for its ability to stimulate lymphocytes and to elevate their FASL expression; especially on T and NKT cells (Dwyer and Johnson, 1981; Takeda et al., 2000; Trautwein et al., 1998) . In order to determine whether FASL expression between NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mouse lines differed, hepatic lymphocytes isolated from these mice 2 hours post Con A injection were stained with anti-mouse FASL monoclonal antibody (FLIM58) and analysed by flowcytometry.

Two hours post Con A administration, FASL was found to be upregulated in βTCR^{+} and βTCR^{-} hepatic cells when compared to vehicle (saline) treated control mice. The expression levels of FASL on βTCR^{+} cells of NOD.*D1Bax208*^{m1} mice was however no greater than that on T cells of NOD.*D1Bax208*^{2} mice (Figure 5.5A). Nevertheless, post Con A injection, the proportions of FASL^{+} T cells in NOD.*D1Bax208*^{m1} mice expanded more than that in NOD.*D1Bax208*^{2} mice, compared to saline treated controls (Figure 5.5B; $p<0.02$; Mann-Whitney U test). The proportions of FASL^{+} βTCR^{+} and βTCR^{-} hepatic cells in NOD.*D1Bax208*^{m1} mice were higher than in NOD.*D1Bax208*^{2} mice (Figure 5.5B; $p<0.04$ in both; Mann-Whitney U test). Saline treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice had

similar numbers of T cells in their livers, however, ConA treatment caused expansion in T cell numbers and this appeared to be greater in NOD.*D1Bax208*^{m2} mice which expressed lower levels of FASL, than in NOD.*D1Bax208*^{m1} mice (Figure 5.5C, $p<0.03$; Mann-Whitney U test). Total numbers of $CD4^+$ T cells in both these ConA treated mouse lines were similar and the difference in T cell numbers appeared to be due to an elevation in $CD8^+$ T cell numbers in NOD.*D1Bax208*^{m2} mice (Figure 5.5C, $p<0.006$; Mann-Whitney U test). There was however no difference in the numbers of non-T cells in these mouse lines post ConA treatment. As activated murine T cells undergoing proliferation are sensitive to FAS/FASL-mediated cell death (Fortner and Budd, 2005; Ju et al., 1995), it was therefore possible that FASL triggered the apoptosis of proliferating FAS^+ T cells in Con A treated mice. The larger pool of FAS ligand expressing cells found in NOD.*D1Bax208*^{m1} mice might have therefore caused a greater contraction of the numbers of stimulated T cells in these mice than in NOD.*D1Bax208*^{m2} mice, which had significantly fewer numbers of $FASL^{hi}$ T cells.

Even though the proportions of FASL expressing hepatic lymphocytes in NOD.*D1Bax208*^{m1} mice were significantly higher than in NOD.*D1Bax208*^{2} mice post 2 hour Con A treatment, difference in CD95L expression levels on βTCR^+ and βTCR^- hepatic cells between the two mouse lines was subtle. Since Con A injection rapidly upregulate membrane FASL expression on murine hepatic NKT cells than on T or NK cells (Takeda et al., 2000), disparity in FASL expression between

NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mouse lines would be more pronounced in this cell type. Flowcytometric analysis revealed similar levels of FAS-ligand expression on NKT cells of saline treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. However, post Con A injection, FASL expression on NKT cells and NKT subsets, ($CD4^+$ and DN cells) of NOD.*D1Bax208*^{m1} mice, which had fewer GATA-1 and 3 transcription factor binding sites, was significantly higher than that on those of NOD.*D1Bax208*^{m2} mice (Figure 5.6C; $p<0.008$; Mann-Whitney U test), an observation consistent with the previously reported repressive effects of GATA-3 on gene expression (Anderson et al., 2002; Imagawa et al., 1997; Taghon et al., 2007). Saline treated control NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice displayed similar hepatic NKT cell proportions and absolute numbers (Figure 5.6A), however 2 hours after the Con A treatment, NOD.*D1Bax208*^{m1} mice had significantly lower numbers of hepatic NKT cells than NOD.*D1Bax208*^{m2} mice (Figure 5.6B; $p<0.008$, Man-Whitney U test). While there was a dramatic diminution in proportions and numbers of NOD.*D1Bax208*^{m1} NKT cells ($p<0.008$ and 0.04 respectively, Man-Whitney U test) and both NKT subsets ($CD4^+$: $p<0.008$ and 0.008 respectively, Man-Whitney U test; DN: $p<0.008$ and 0.04 respectively, Man-Whitney U test), the NKT cell proportions remained similar in NOD.*D1Bax208*^{m2} mice. As activated mouse NKT cells *in vitro* can undergo apoptosis via FAS/FASL (Leite-de-Moraes et al., 2000), it is therefore possible that enhanced apoptosis of activated NKT cells occurred in Con A treated NOD.*D1Bax208*^{m1} mice owing to their higher level of FASL expression.

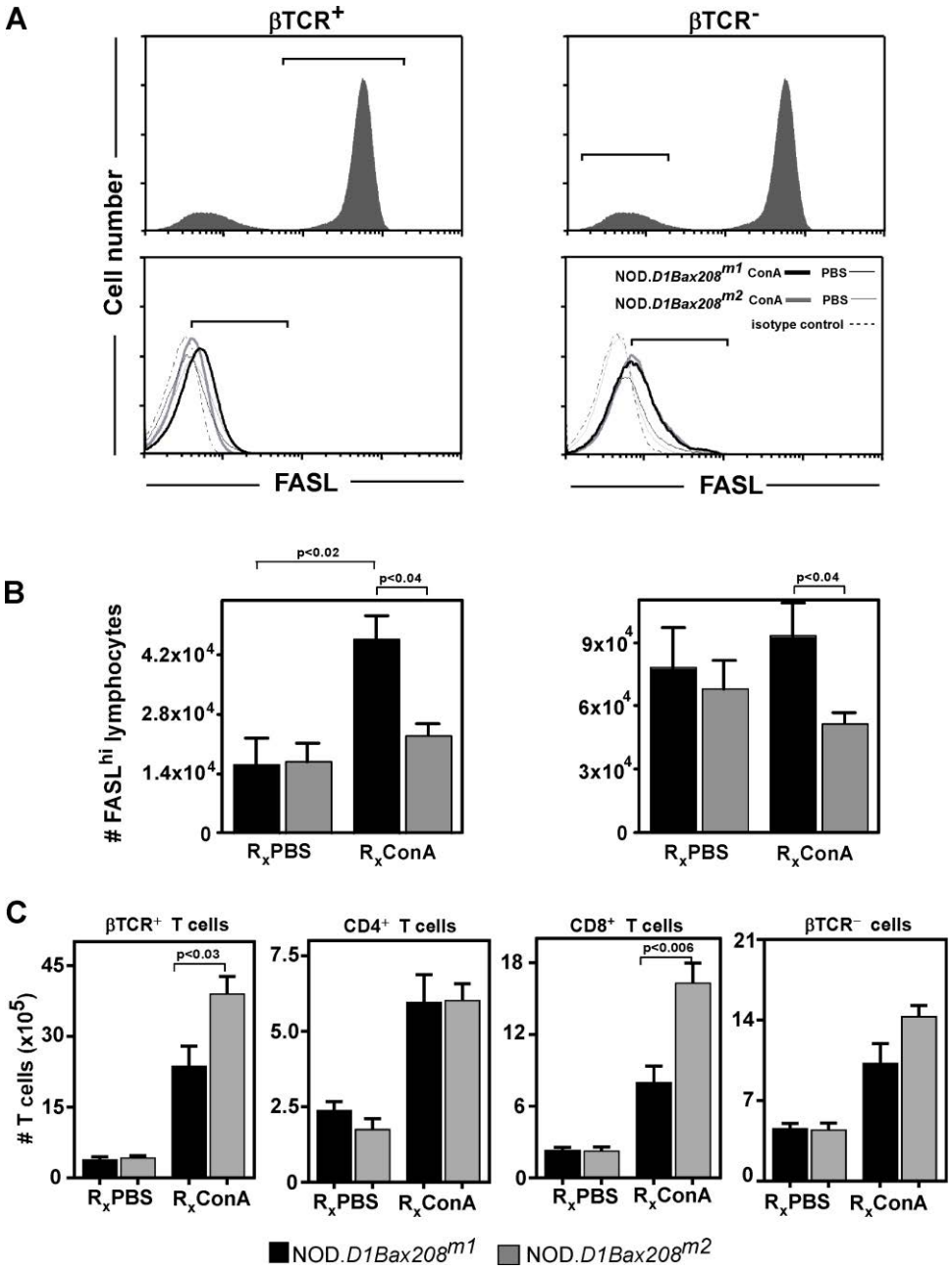


Figure 5.5. Difference in FASL expression on hepatic βTCR$^+$ and βTCR$^-$ cells from Con A treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Lymphocyte subsets were electronically gated applying the strategy as shown in panel A. Histograms(B) demonstrating numbers of FASL expressing T cells and non-T cells from saline and Con A treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Histograms (C) representing the total numbers of T and non-T cells from saline and Con A treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. n=5, statistical analysis performed using Mann-Whitney U test.

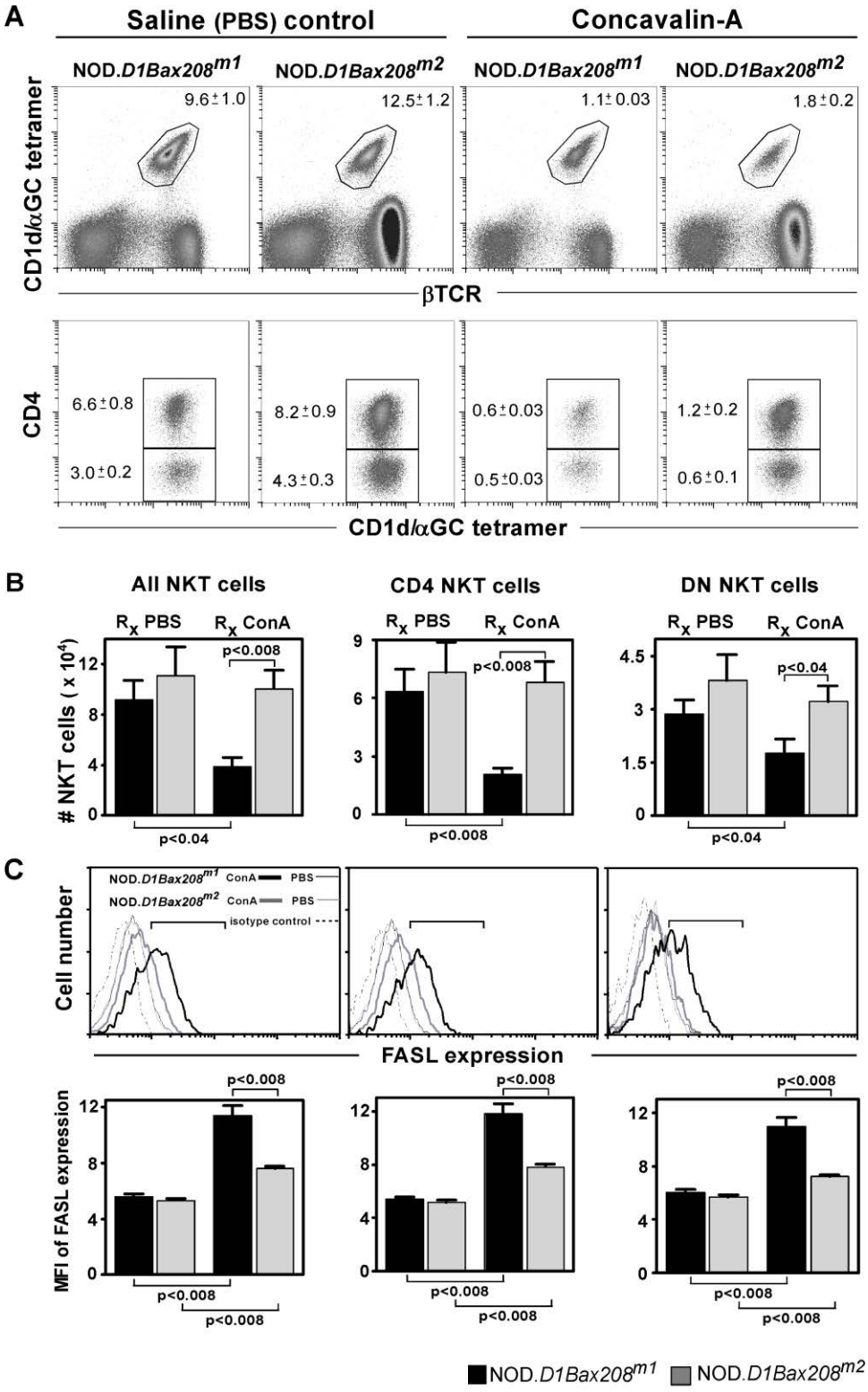


Figure 5.6 Difference in hepatic NKT cell numbers and FASL expression in Con A treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. NKT cell subsets were electronically gated applying the strategy as shown in panel (A). *(figure legend continued next page)*

Histograms (B) represents the absolute numbers of NKT cells and their CD4+ and DN subsets from Con A and saline treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Histograms (C) illustrates the Mean Fluorescence Intensity of FASL expression on CD1d-αGalCer tetramer^{+}TCR^{+} NKT cells and their CD4^{+} and Double Negative (DN) subsets and from Con A and saline treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. n=5, statistical analysis performed using Mann-Whitney U test.

5.2.3 Diabetes Incidence in NOD.*D1Bax208*^{m1} & NOD.*D1Bax208*^{m2} mice

Flow cytometric analysis revealed a variation in FASL expression in NOD mice carrying the mutant alleles of *D1Bax208* (Figures 5.5 and 5.6). Female NOD mice bearing a loss-of-function mutation of *Fasl* were totally protected from T1D (Anderson and Bluestone, 2005; Su et al., 2000) and defects in the FAS-signalling protect against EAE in this animal model of multiple sclerosis (Sabelko et al., 1997; Waldner et al., 1997). The blockade of the FAS/FASL pathway therefore appears to have a protective effect against organ-specific autoimmune diseases. Hence the reduced FASL expression in NOD.*D1Bax208*^{m2} could possibly confer resistance against diabetes in these mice as opposed to the mutant NOD.*D1Bax208*^{m1} mice, which showed comparatively higher FASL expression. To examine whether the allelic FASL expression had any effect on the diabetes phenotype on the NOD background, blood glucose levels of cohorts of NOD.*D1Bax208*^{m1} (42 female and 39 male mice) and NOD.*D1Bax208*^{m2} (41 female and 15 male mice) were monitored from 8 weeks to 32 weeks of age, and the incidence of diabetes compared with that of female and male NOD/Lt mice (n= 37 & 22 respectively, Figures 5.7A and 5.7B).

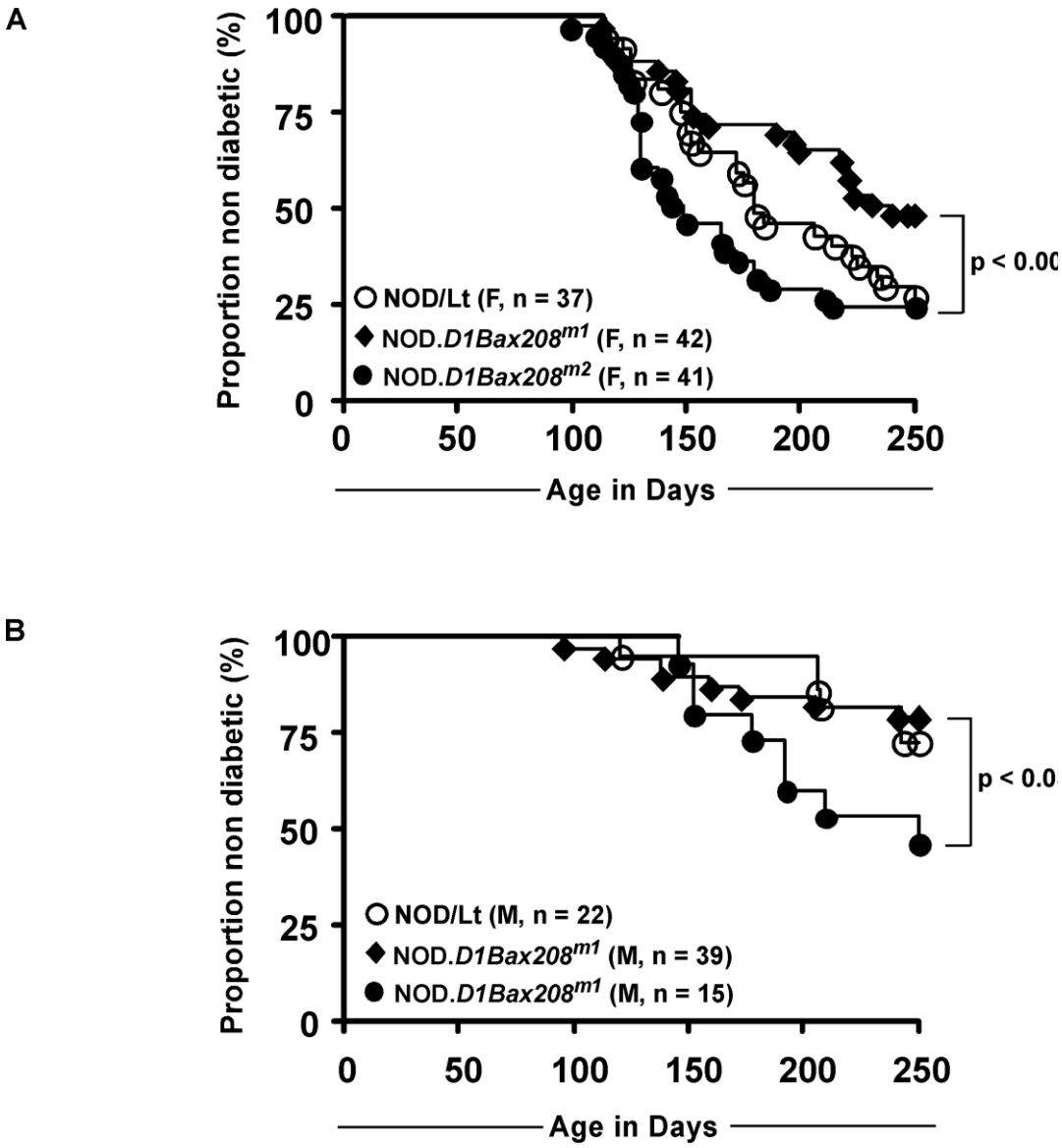


Figure 5.7 Life table analysis of spontaneous diabetes in NOD/Lt, NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Incidence of spontaneous T1D in female (A) and male (B) NOD/Lt (empty circles), NOD.*D1Bax208*^{m1} (diamonds) and NOD.*D1Bax208*^{m2} (filled circles) mice.

NOD mice carrying the mutant alleles of *D1Bax208* displayed significant differences in their diabetes incidence, while an intermediate disease incidence was noted in the control NOD/Lt mice. Contrary to the proposed protective effect of low FASL expression in autoimmune diabetes, more NOD.*D1Bax208*^{m2} mice (which exhibited lower levels of FASL expression as opposed to NOD.*D1Bax208*^{m1} mice) became diabetic compared to NOD.*D1Bax208*^{m1} mice (31/41 (76%) vs. 21/42 (50%) in case of females; $p<0.003$, life table analysis; $p<0.03$, Fisher's exact test and 8/15 (53%) vs. 8/39 (21%) in case of males; $p<0.03$, life table analysis; $p<0.05$, Fisher's exact test).

5.2.4 SNP typing NOD.*D1Bax208*^{m1} & NOD.*D1Bax208*^{m2} mice

The difference in diabetes incidence observed in NOD lines carrying the mutant alleles of *D1Bax208* was likely an outcome of their allelic FASL expression; however, there existed a possibility that background genetic contamination in either mouse line could have contributed to this effect. 689 single nucleotide polymorphisms (SNPs) from NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2} and control NOD mice were genotyped (Australian Genome Resource Facility, Melbourne, Australia) in order to confirm the genetic homogeneity of these mice lines. Except for four markers linked to the killer cell lectin-like receptor subfamily B member 1C (*Klrb1c*/CD161/NK1.1; between 91414637-117084506 bases on chromosome 6) in NOD.*D1Bax208*^{m1} mice (Table 5.3), the three mice lines appeared to be genetically identical.

Chr#	Position	Marker	NOD.*D1Bax208*^{m1}	NOD. *D1Bax208*^{m2}	NOD/Lt
6	91414637	rs13478887	G	A	A
6	103501481	rs13478939	G	A	A
6	109597329	rs13478963	T	G	G
6	117084506	rs13478991	C	A	A

Table 5.3 Genetic variation was noted in NOD.*D1Bax208*^{m1} mice line. Comparison of SNP typing revealed nucleotide dissimilarity at positions linked to *Klrb1c* on chromosome 6 in DNA samples from NOD.*D1Bax208*^{m1} mice (n=5) when compared to samples from NOD.*D1Bax208*^{m2} line (n=5) and the control NOD/Lt strain (n=5). As for the remaining 685 SNPs spanning the genome, the three lines were identical.

To exclude the possibility that the *Klrb1c* allele affected autoimmune diabetes phenotype in NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice, these strains were backcrossed with the parental NOD/Lt strain and the F_1 mice intercrossed. The NK1.1 allele present on the NOD.*D1Bax208*^{m1} intercross (IC) progeny carrying *Klrb1c,* selectively expressed on the surface of their NK and NKT cell subsets, was identified by means of flowcytometry on peripheral blood lymphocyte samples stained with mouse anti-CD161 monoclonal antibody (Figure 5.8).

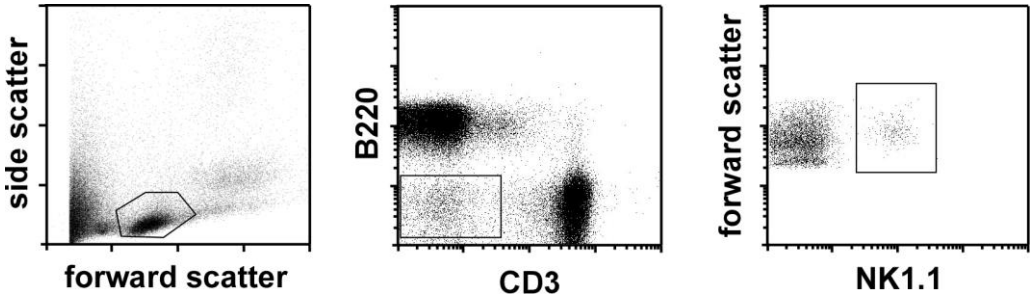


Figure 5.8 NOD.*D1Bax208*^{m1} mouse expressing the NK1.1 marker. NOD.*D1Bax208*^{m1} mice having cells expressing NK1.1 marker were identified by applying the gating strategy as shown. These mice were excluded from the breeding program.

Mice homozygous or heterozygous for NK1.1 were excluded while male and female mice in which the NK1.1 allele was absent, but were homozygous for the 167bp allele at the *D1Bax208* locus, were selected and mated to re-establish NOD.*D1Bax208*^{m1} line. Similarly NOD.*D1Bax208*^{m2} IC progeny homozygous for the 179bp allele at the *D1Bax208* locus were selected and mated to re-establish the NOD.*D1Bax208*^{m2} line. In both cases the PCR results were validated using High Resolution Melt (HRM) analysis (Figure 5.9 and 5.10). Genetic homogeneity of the re-established NOD lines carrying the mutant alleles of *D1Bax208* was confirmed by typing 687 SNPs (Australian Genome Resource Facility, Melbourne, Australia), none of which differed from the NOD/Lt parental strain (Table 5.4).

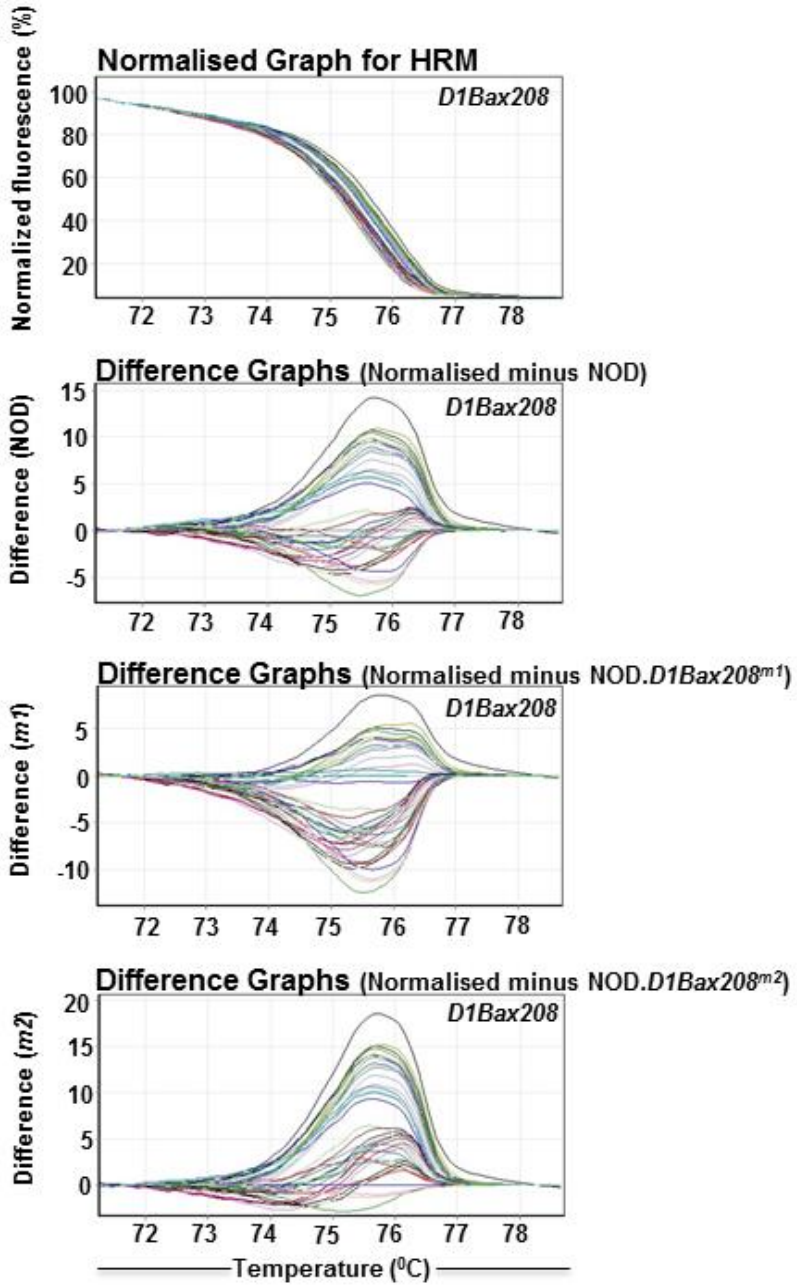


Figure 5.9 High Resolution Melt analysis of *D1Bax208* locus distinguishing NOD/Lt, NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} genotypes. Representative difference graphs compares the melting curves of test DNA sample (each represented by the different coloured curves) with a given genotype control (*viz.* DNA samples of NOD/Lt, NOD.*D1Bax208*^{m1} or NOD.*D1Bax208*^{m2}). The samples with similar melting curves (confidence threshold > 85%) were automatically clustered into groups in the post-PCR HRM analysis as explained in figure 5.10. HRM analysis performed on a Rotor-Gene 6000 series real time analyser.

No.	Colour	Sample Name	Genotype	Confidence %
1		Sample 1	NOD	95.84
2		Sample 2	NOD	94.55
3		Sample 3	Variation	<85
4		Sample 4	Variation	<85
5		Sample 5	NOD	93.46
6		Sample 6	NOD	94.87
7		Sample 7	Variation	<85
8		Sample 8	Variation	<85
9		Sample 9	Variation	<85
10		Sample 10	NOD	97.94
11		Sample 11	Variation	<85
12		Sample 12	NOD	96.06
13		Sample 13	NOD	94.73
14		Sample 14	*D1Bax208*^{m1}	93.37
15		Sample 15	*D1Bax208*^{m1}	95.57
16		Sample 16	*D1Bax208*^{m1}HET	95.44
17		Sample 17	NOD	94.93
18		Sample 18	*D1Bax208*^{m1}HET	87.14
19		Sample 19	Variation	<85
20		Sample 20	Variation	<85
21		Sample 21	NOD	98.95
22		Sample 22	*D1Bax208*^{m1}	86.80
23		Sample 23	*D1Bax208*^{m1}HET	89.80
24		Sample 24	*D1Bax208*^{m1}HET	93.75
25		Sample 25	*D1Bax208*^{m1}HET	99.02
26		Sample 26	*D1Bax208*^{m1}	88.12
27		Sample 27	*D1Bax208*^{m1}	89.76
28		Sample 28	*D1Bax208*^{m1}HET	98.12
29		Sample 29	*D1Bax208*^{m1}	98.24
30		Sample 30	*D1Bax208*^{m1}	98.63
31		Sample 31	*D1Bax208*^{m1}	99.25
32		Sample 32	*D1Bax208*^{m2}	89.47
33		Sample 33	NOD	96.36
34		Sample 34	*D1Bax208*^{m1}HET	99.51
35		Sample 35	*D1Bax208*^{m2}	97.55
36		Sample 36	*D1Bax208*^{m1}HET	99.47
37		Sample 37	*D1Bax208*^{m1}	99.6
38		*D1Bax208*^{m1}HET PC	*D1Bax208*^{m1}HET	100.00
39		*D1Bax208*^{m1} PC	*D1Bax208*^{m1}	100.00
40		*D1Bax208*^{m2} PC	*D1Bax208*^{m2}	100.00
41		NOD PC	NOD	100.00

Figure 5.10 Genotypes clearly distinguished by HRM analysis of *D1Bax208* locus. Melt curves of test samples were compared with positive control (PC). Samples with confidence threshold above 85% in their difference graphs were automatically assigned the genotypes of NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2} or NOD/Lt by the Rotor-Gene 6000 Series Software v1.7.

Chr#	Position	Marker	NOD.*D1Bax208*^{m1}	NOD. *D1Bax208*^{m2}	NOD/Lt
6	91414637	rs13478887	A	A	A
6	103501481	rs13478939	A	A	A
6	109597329	rs13478963	G	G	G
6	117084506	rs13478991	A	A	A

Table 5. 4 SNP analysis of the re-established NOD lines carrying the mutant alleles of *D1Bax208.* Confirmation of genetic homogeneity of the re-established NOD lines carrying the mutant alleles of *D1Bax208*. n=5 for all three mouse lines.

5.2.5 Increased diabetes incidence in rederived NOD.*D1Bax208*^{m2} mice

The difference in diabetes incidence observed in the NOD lines carrying the mutant alleles of *D1Bax208* could have been an outcome of the background genetic contamination present in NOD.*D1Bax208*^{m1} line. In order to verify the preliminary findings, diabetes incidence study was repeated in cohorts of re-established female and male NOD.*D1Bax208*^{m1} (n=26 and 22 respectively) and NOD.*D1Bax208*^{m2} (n= 24 and 26 respectively) mouse lines (Figures 5.11 A and 5.11 B). Consistent with the earlier findings in NOD mice carrying the mutant alleles of *D1Bax208*, the re-established mouse lines also significantly differed in their diabetes incidence.

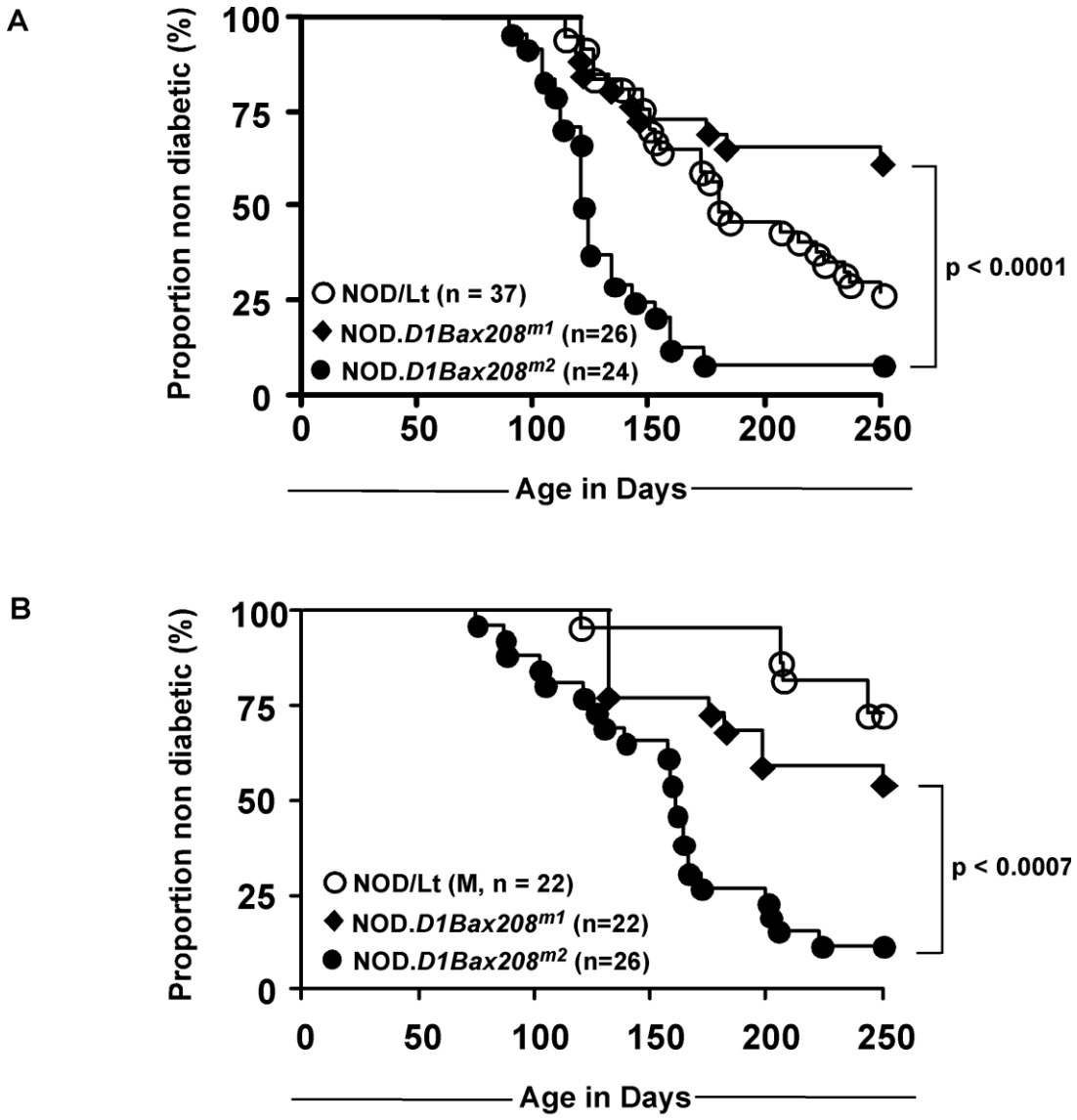


Figure 5.11 Life table analysis of spontaneous diabetes in NOD/Lt, NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Incidence of spontaneous T1D in female (A) and male (B) NOD/Lt (empty circles), NOD.*D1Bax208*^{m1} (diamonds) and NOD.*D1Bax208*^{m2} (filled circles) mice.

Mice of either sex of the low-FASL-expressing NOD.*D1Bax208*^{m2} line displayed significantly higher incidence of the disease when compared to NOD.*D1Bax208*^{m1} mice (22/24 (92%) vs. 10/26 (38%) in case of females; $p<0.0001$, life table analysis; $p<0.0002$, Fisher's exact test, and 23/26 (88%) vs. 10/22 (45%) in case of males; $p<0.0007$, life table analysis; $p<0.002$, Fisher's exact test).

5.2.6 Severity of insulitis in NOD.*D1Bax208*^{m1} & NOD.*D1Bax208*^{m2} mice

NOD$^{gld/+}$ mice or wild type NOD mice treated with FASL-neutralizing antibody (MFL4), remained relatively free of insulitis (Kim et al., 2000; Kim et al., 1999; Mohamood et al., 2007; Su et al., 2000), indicating that a variation in FASL expression can affect the rates of islet-cell destruction. In order to examine whether the allelic FASL expression noted in NOD mice carrying the mutant alleles of *D1Bax208* affected the severity of insulitis, histological analysis was performed on the pancreata of 36-wk-old female and male NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. While the pancreatic islets of NOD.*D1Bax208*^{m1}mice, which expressed higher levels of FASL, were moderately infiltrated by leukocytes, severe insulitis and extensive destruction were observed in majority of islets of the NOD.*D1Bax208*^{m2} mice (Figure 5.12 A and 5.12 B, Table 5.5), indicating that the death receptor ligand played a major role in inhibiting islet infiltration in NOD mice.

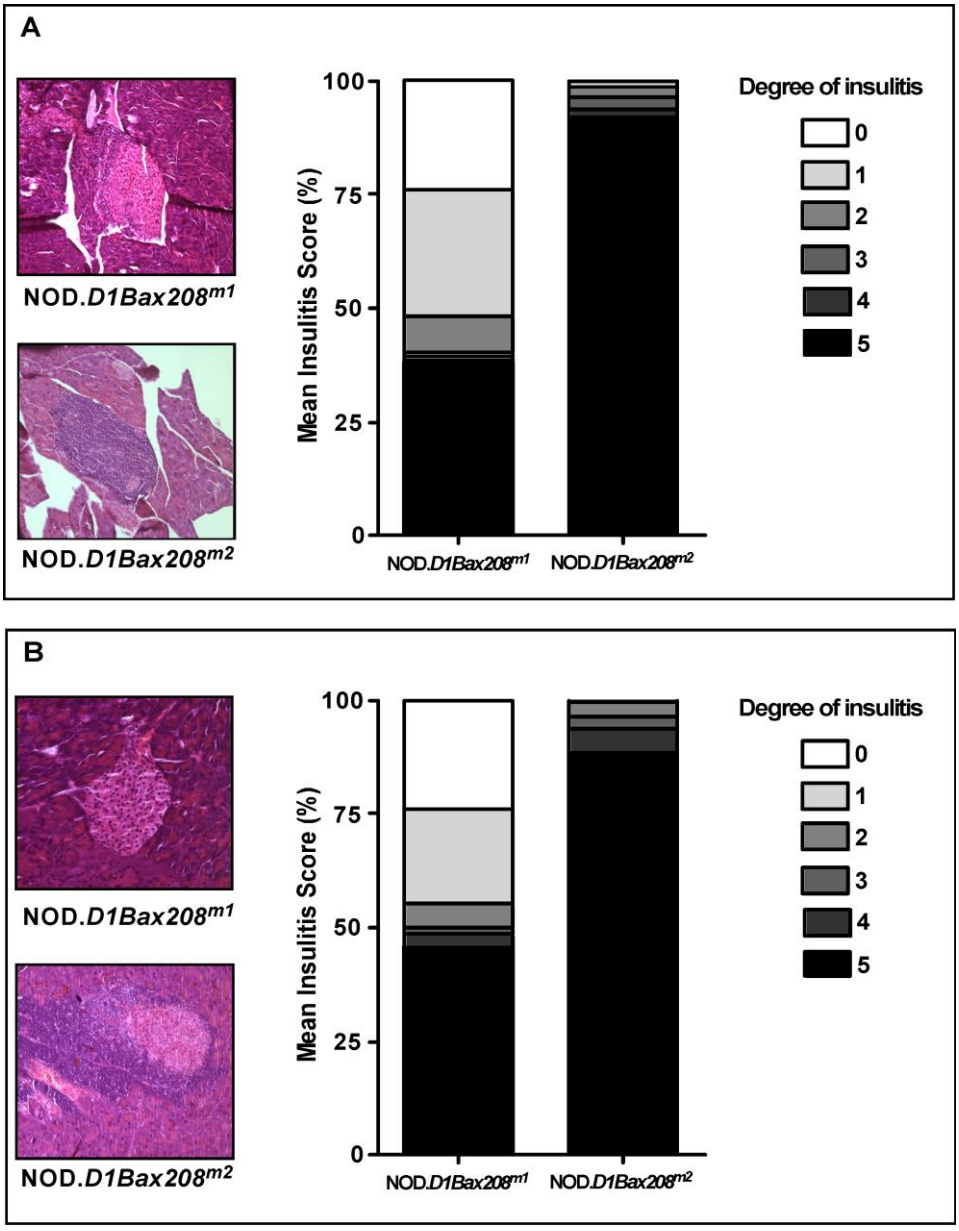


Figure 5.12 Histological analysis of H&E stained pancreata of NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Analysis of severity of insulitis in female (A) and male (B) NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice at 36 week of age. Islets of H&E stained pancreata were scored for the degree of insulitis on a scale of 0-5: 0, no infiltrate; 1, periislet (<25%); 2, 25-50% accumulation of inflammatory cells; 3, 50-75% intraislet infiltration; 4, severe structural derangement and complete loss of cells; 5, diabetic. NOD.D1Bax208m1 mice had more islets with lower degrees of insulitis (scales 0 and 1), while islets with higher degrees if infiltration (scale 2-5) were significantly higher in NOD.*D1Bax208*^{m2} mice.

Scale of insulitis	0	1	2	3	4	5
NOD.*D1Bax208*^{m1}	39	45	13	1	2	38
NOD.*D1Bax208*^{m2}	3	16	27	33	21	92
p	0.0001	0.0001	0.0208	0.0001	0.0001	0.0001
NOD.*D1Bax208*^{m1}	44	38	10	2	6	88
NOD.*D1Bax208*^{m2}	0	6	25	23	46	45
p	0.0001	0.0001	0.0085	0.0001	0.0001	0.0001

Table 5.5 The proportion of islets exhibiting various degrees of insulitis expressed as a percentage of the total number of islets scored. Islets of H&E stained pancreata were scored for the degree of insulitis on a scale of 0-5: 0, no infiltrate; 1, periislet infiltrate (<25%); 2, 25-50% accumulation of inflammatory cells; 3, 50-75% intraislet infiltration; 4, severe structural derangement and complete loss of cells; 5, diabetic. Insulitis scores for female and male NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice are depicted on the upper and lower panels respectively. Comparison of NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} insulitis scores performed by Fisher's exact test.

5.2.7 Peripheral T cell compartment in NOD.*D1Bax208^{m1}* and NOD.*D1Bax208^{m2}* mice

Based on these data, we therefore hypothesise that the disparity in GATA binding site numbers in *D1Bax208* (immediately 3' to *FasL*) in NOD.*D1Bax208^{m1}* and NOD.*D1Bax208^{m2}* mice affected their incidence of diabetes via effects on lymphocyte FASL expression. Modulation of FASL expression on lymphocytes can influence their ability to trigger apoptosis (Kayagaki et al., 1997). Mice with missense mutations in *Fas* or *Fasl* developed acute lymphoproliferative disorders, characterized by progressive accumulation of abnormal double negative T cells ($\alpha\beta$TCR$^+$, CD4$^-$, CD8$^-$, Thy1$^+$ and B220$^+$), development of lymphadenopathy, splenomegaly and spontaneous production of autoantibodies to nuclear antigens (Adachi et al., 1996; Takahashi et al., 1994; Watanabe-Fukunaga et al., 1992). Autoimmune lymphoproliferative syndrome (ALPS), a similar systemic autoimmune condition in man, was identified in individuals who had a mutation in *FAS* coding sequence (Fisher et al., 1995). These genetic autoimmune disorders were characterized by polyclonal expansion of abnormal lymphocytes due to a defect in apoptosis, suggesting a crucial role for FAS and its ligand in preserving peripheral tolerance. We therefore sought to determine if the allelic FAS ligand expression in NOD mice carrying the mutant alleles of *D1Bax208* resulted in a functional phenotype. In order to determine whether variation in FASL expression had any effect on activated T cell survival, proportions of activated (CD4$^+$CD44$^+$CD62L$^-$), memory (CD4$^+$CD44^{int}CD62L^{int}) and naïve

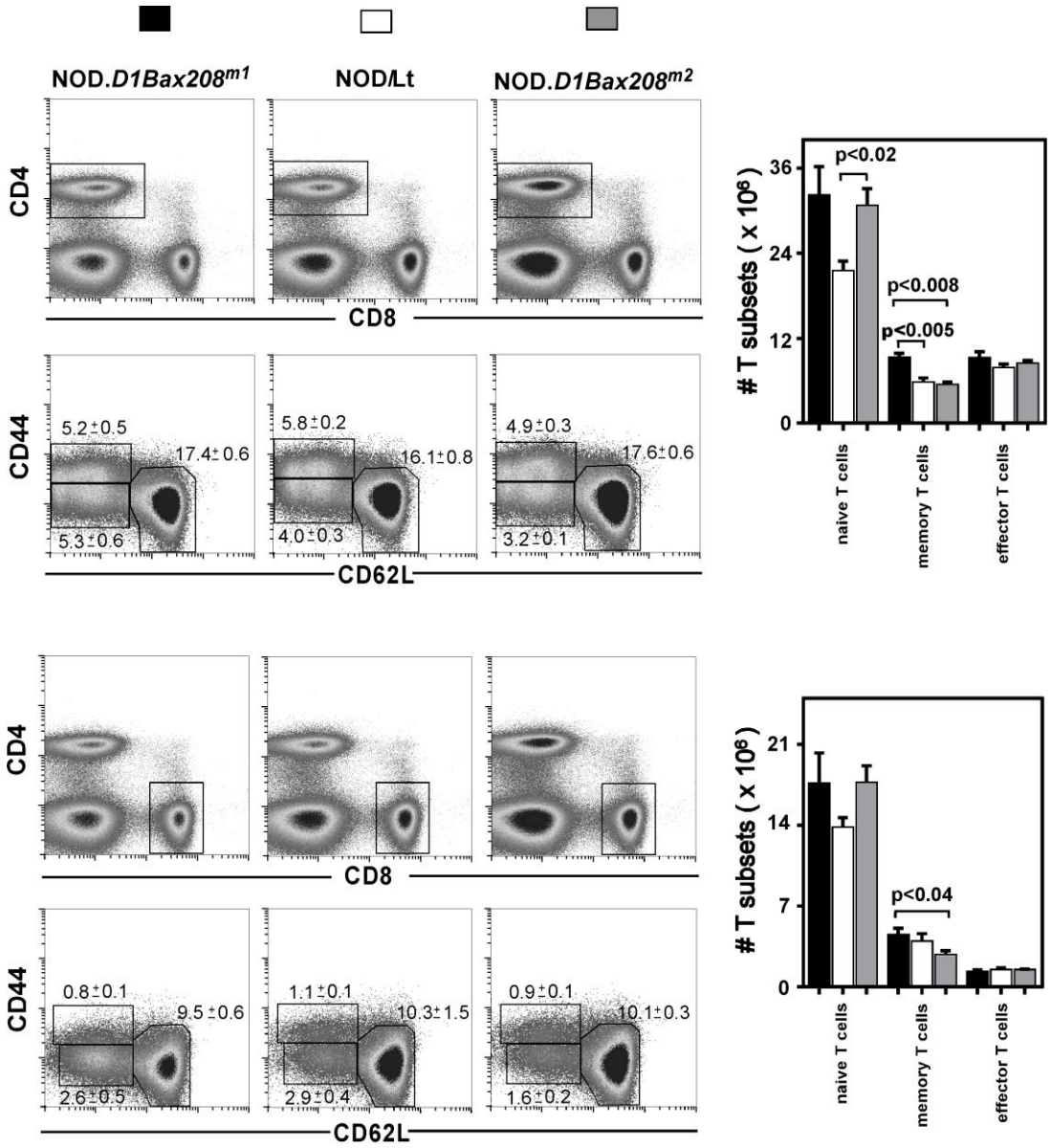


Figure 5.13 Splenic T cell subsets in NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Representative plots of proportions of $CD4^+CD44^{low}CD62L^+$ ("naïve"), $CD4^+CD44^+CD62L^-$ ("effector") and $CD4^+CD44^{int}CD62L^{int}$ ("memory") T cells in spleens of female NOD.*D1Bax208*^{m1} (n=5; black), NOD/Lt (n =5; white) and NOD.*D1Bax208*^{m2} (n=5; grey) mice. $CD4^+$ and $CD8^+$ T cell subset proportions are shown in the plots (mean ± SEM) and the absolute numbers (mean ± SEM) represented by histograms. Significance by Mann-Whitney U test; p values are not corrected for multiple hypothesis testing.

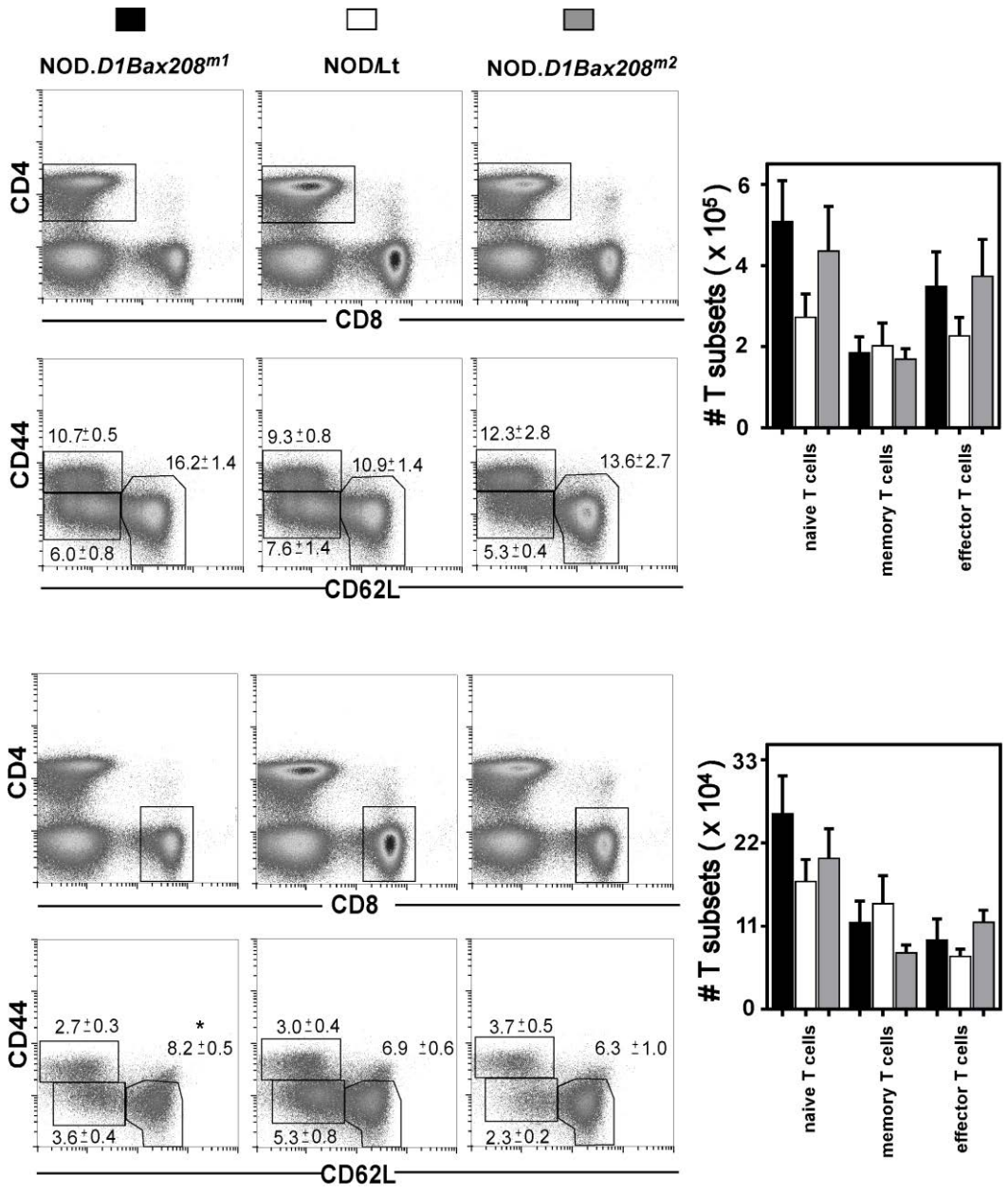


Figure 5.14 Hepatic T cell subsets in NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Representative plots of proportions of $CD4^+CD44^{low}CD62L^+$ ("naïve"), $CD4^+CD44^+CD62L^-$ ("effector") and $CD4^+CD44^{int}CD62L^{int}$ ("memory") T cells in spleens of female NOD.*D1Bax208*^{m1} (n=5; black), NOD/Lt (n =5; white) and NOD.*D1Bax208*^{m2} (n=5; grey) mice. $CD4^+$ and $CD8^+$ T cell subset proportions are shown in the plots (mean ± SEM) and the absolute numbers (mean ± SEM) represented by histograms. Significance by Mann-Whitney U test; p values are not corrected for multiple hypothesis testing.

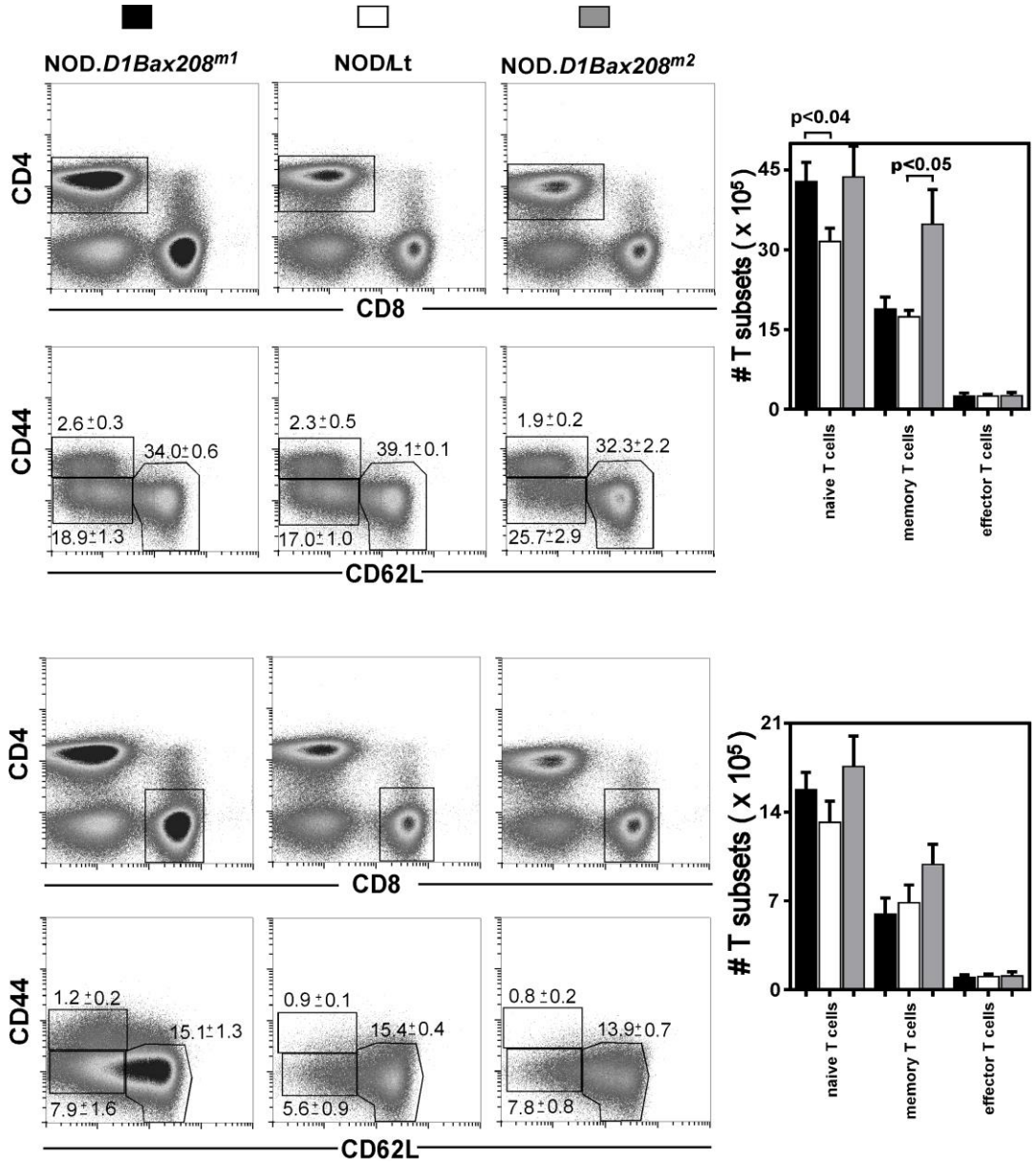


Figure 5.15 T cell subsets in the lymph nodes of NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. Representative plots of proportions of CD4^{+}CD44^{low}CD62L^{+} ("naïve"), CD4^{+}CD44^{+}CD62L^{-} ("effector") and CD4^{+}CD44^{int}CD62L^{int} ("memory") T cells in spleens of female NOD.*D1Bax208*^{m1} (n=5; black), NOD/Lt (n =5; white) and NOD.*D1Bax208*^{m2} (n=5; grey) mice. CD4^{+} and CD8^{+} T cell subset proportions are shown in the plots (mean ± SEM) and the absolute numbers (mean ± SEM) represented by histograms. Significance by Mann-Whitney U test; p values are not corrected for multiple hypothesis testing.

($CD4^+CD44^{low}CD62L^+$) phenotype $CD4^+$ and $CD8^+$ T cells in the spleens, livers and lymph nodes of pre-diabetic female NOD mice carrying the mutant alleles of *D1Bax208* were compared to those of parental NOD/Lt mice. A flow cytometric analysis failed to identify any robust differences after correction for multiple hypotheses testing (Bonferroni multiple comparison test, Figures 5.13, 5.14 and 5.15 respectively). This result eliminated the possibility of a lymphoaccumulation-mediated exacerbation of diabetes in NOD.*D1Bax208*^{m2} mice.

5.2.8 Reciprocal adoptive transfer of diabetogenic splenocytes into irradiated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice.

Although variation in the level of FASL expression was insufficient to cause quantitative differences in T cells of NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice, it was possible that it could affect the diabetogenic activity of T cells via some other mechanism (Su et al., 2000). To determine whether the decreased FASL-expression in the hematopoietic compartment of female NOD.*D1Bax208*^{m2} mice played a role in enhancing its autoimmunity, the diabetogenic activity of splenocytes from these mice were compared to that of splenocytes from the parental NOD/Lt mice in adoptive transfer into 8-10 week old female NOD.*scid* recipients. Irrespective of the donor, diabetes occurred in all the recipient mice within five-week post transfer (Figure 5.16 A), suggesting a limited intrinsic role for FASL in regulating the diabetogenicity of splenocytes. To examine the possibility that the enhanced beta cell destruction seen in NOD.*D1Bax208*^{m2} mice

was a non-hematopoietic trait, reciprocal spleen cell transfers into irradiated recipients were performed between NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. On transferring diabetogenic splenocytes from female NOD.*D1Bax208*^{m1} mice, diabetes occurred in all 13 NOD.*D1Bax208*^{m2} recipients within 4 weeks, while a significant delay (> 14 weeks) occurred in the 10 NOD.*D1Bax208*^{m1} recipients ($p<0.0001$; life table analysis, Figure 5.16 B). While NOD.*D1Bax208*^{m2} recipients (n=6) of diabetogenic splenocyte from NOD.*D1Bax208*^{m2} mice had a median survival of 19 days, NOD.*D1Bax208*^{m1} recipients (n=8) of the same donor had a slower progression of the disease, with a median survival rate of 43.5 days, suggesting that accelerated diabetes was associated with low-FASL-expression in NOD.*D1Bax208*^{m2} recipients. The fact that, the difference in survival between NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} recipients of diabetogenic splenocyte from NOD.*D1Bax208*^{m2} mice was not statistically significant ($p=0.063$; life table analysis; Figure 5.16 B), suggested a minor intrinsic role for FASL signalling. To summarise, accelerated diabetes was associated with low-FASL-expressing recipients, and to a lesser extent, with low-FASL-expressing donors. This raised the possibility that expression of this death ligand in the nonhematopoietic compartment confers a protective effect against autoimmune diabetes in NOD mice through some previously unidentified mechanism/(s). Also evident was an additional minor intrinsic role for FASL in regulating the diabetogenicity of splenocytes. Severe insulitis and extensive destruction observed in the pancreatic islets of low FASL expressing NOD.*D1Bax208*^{m2} mice is reminiscent of the florid lymphoid infiltrates

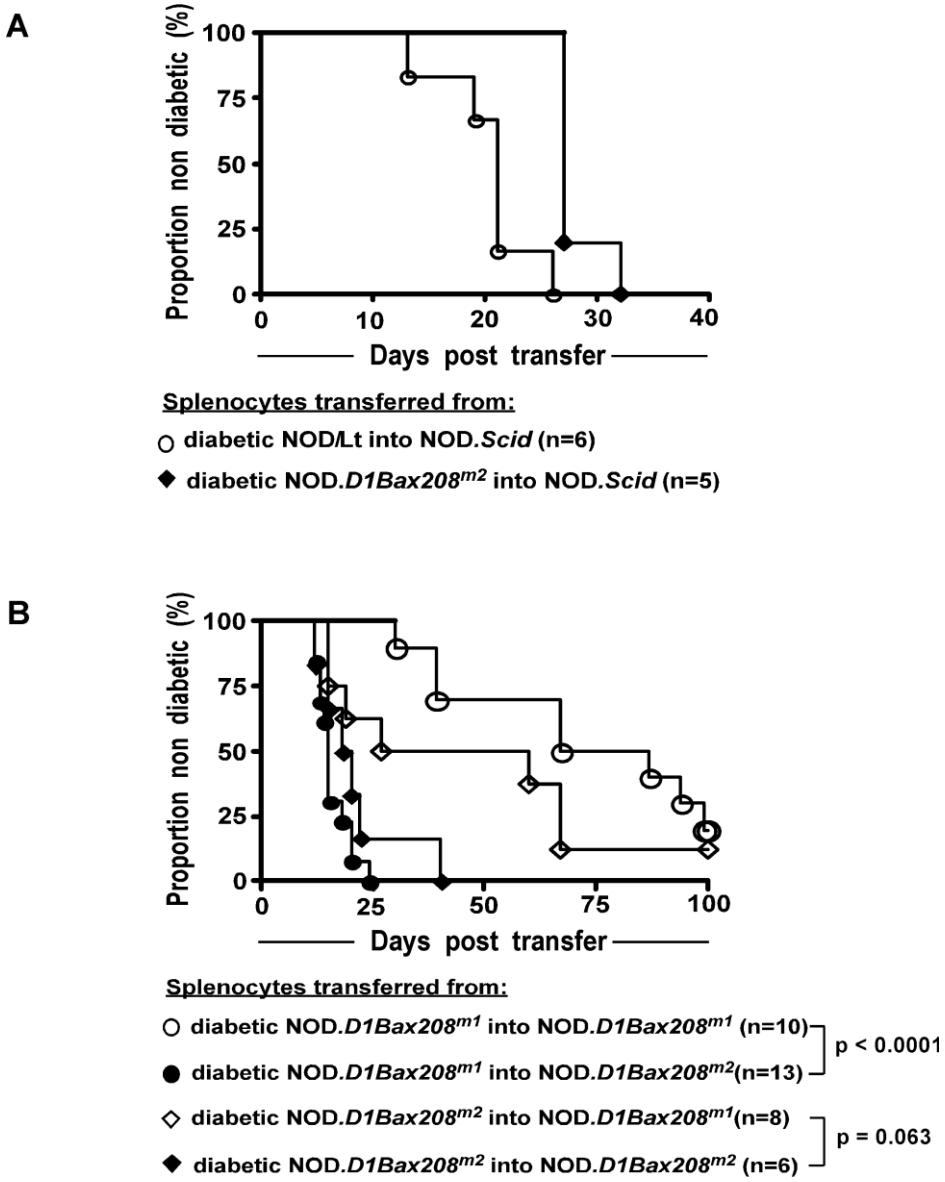


Figure 5.16 Adoptive transfer of diabetes into NOD.*scid* mice (A).Development of diabetes following reciprocal adoptive transfer of splenocytes into irradiated recipients between mice carrying the mutant alleles of *D1Bax208* (B). Pooled splenocytes from diabetic female NOD.*D1Bax208*^{m1} mice transferred into irradiated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} recipients (white circles and black circles respectively) and NOD.*D1Bax208*^{m2} splenocytes into irradiated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} recipients (white diamonds and black diamonds respectively). Statistical comparison performed by life table analysis.

I found in NOD mice that lacked TRAIL (Chapter 4). Moreover, results of the reciprocal adoptive transfer experiments raised the possibility that expression of TRAIL and FASL in the nonhematopoietic compartment confers a protective effect against autoimmune diabetes. TRAIL-signaling exerted inhibitory effects on chemokine production in the islets (Figure 4.22). Considering that among all TNF superfamily members, FASL is most homologous to TRAIL, it was possible that FASL-signaling affected the islets in ways similar to that of TRAIL.

5.2.9 Microarray analysis of islet transcripts

In order to identify differentially expressed cytokines or chemokines, whole islets from preinsulitic (3 week old) NOD.*D1Bax208*^{m1} mice (n=6) and NOD.*D1Bax208*^{m2} mice (n=5) were isolated by our collaborator Stacey Walters (Shane T Grey's laboratory at the Department of Gene Therapy and Autoimmunity, Garvan Institute of Medical Research, NSW) for expression profiling. Margaret Jordan extracted RNA from the islets and performed the microarray analysis. Applying a Mann-Whitney U threshold of zero (no overlap between groups), we identified 1107 highly differentially expressed genes in the islets of NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice. This analysis identified the downregulation of *Socs1* in NOD.*D1Bax208*^{m2} mice. SOCS1 (suppression of cytokine signalling) binds to cytokine-receptors and Janus-activated Kinases (JAK), and attenuate the pathogenic effects of cytokines, especially IFN-γ. Mice with mutations and deletion of *Socs1* had excessive IFN-γ responses and manifested

mutations and deletion of *Socs1* had excessive IFN-γ responses and manifested extensive IFN-γ dependent pathology (Alexander et al., 1999; Marine et al., 1999). This finding suggested that β-cell protective effects of FASL was possibly mediated by SOCS1 suppressing IFN-γ-induced islet damage.

5.3 Discussion

Type 1 diabetes results from a systemic breakdown in central and peripheral tolerance mechanisms, leading to expansion of autoreactive T cells that attack and destroy the insulin-producing β-cells in pancreatic islets. FAS ligand is a key death receptor in the extrinsic apoptotic pathway (Nagata and Golstein, 1995). The FAS/FASL system, through apoptosis, provides an effective self-regulatory mechanism to eliminate autoreactive cells and maintain homeostasis (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995; Nagata and Suda, 1995).

5.3.1 GATA transcription factors affect FASL expression.

The *D1Bax208* locus is a simple sequence repeat which lies between and immediately 3' to *Fasl* and a putative glutamic pyruvic transaminase gene *Gm5049.* In NOD mice, it was found to be highly mutable. The degree of variation in repeat numbers at the *D1Bax208* locus was unlikely to be due to genetic contamination, as none of the 51 other polymorphic markers on chromosome 1 or 636 markers tested on the rest of the autosomes showed any variability (Table 4.4). Out of the three alleles identified at the *D1Bax208* locus, the largest disparity in genetic repeat length was observed between NOD.*D1Bax208*^{m1} (on NOD line carrying the 167bp allele) and NOD.*D1Bax208*^{m2} (on NOD line carrying the 179bp allele) mouse lines (Figure 5.2 and Table 5.1). It was therefore anticipated that functional phenotypes, if any, resulting from allelic differences at *D1Bax208*, were more likely

to be identified in comparisons of these mice. The *D1Bax208* microsatellite consisted of tandem GATA repeats, sequence analysis of which revealed putative binding sites for the transcription factors GATA-1, GATA-3 and GATA-4. Previous studies indicate that GATA-1 or GATA-3 could act as both activators and repressors of transcription (Cheng et al., 2009; Fujiwara et al., 2009; Wei et al., 2011; Yu et al., 2009). Owing to *D1Bax208* allelic variation, NOD.*D1Bax208*^{m2} mice had 16% more putative GATA binding sites than NOD.*D1Bax208*^{m1} mice at this locus. To determine whether differences in the numbers of putative GATA binding sites in these mice is of functional significance, FASL expression on hepatic lymphocytes in these mice was compared 2 hours after Con A treatment. The proportions of FASL-expressing hepatic lymphocytes in NOD.*D1Bax208*^{m1} mice were significantly higher than in NOD.*D1Bax208*^{2} mice. The disparity in CD95L expression levels on hepatic βTCR^{+} and βTCR^{-} lymphocytes between the two mouse lines, however, was subtle. Evidence of any disparity in FASL expression between Con A treated NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice was likely to be more pronounced on their NKT cells since Con A injection rapidly upregulate membrane FASL expression on murine hepatic NKT cells than on T or NK cells (Takeda et al., 2000). Consistent with this assumption, FASL expression levels on NKT cells from NOD.*D1Bax208*^{m1} mice, which had fewer numbers of GATA-1 and 3 transcription factor-binding sites, was significantly higher than that on NKT cells from NOD.*D1Bax208*^{m2} mice. These findings suggest an inhibitory role for GATA transcription factors on FASL transcription, an observation consistent with

previously reported repressive effects of GATA-3 on gene expression (Anderson et al., 2002; Imagawa et al., 1997; Taghon et al., 2007). In a recent genome-wide analysis, GATA-3 was found to affect the expression of 623 genes in mouse T cells, and the deletion of *Gata-3* resulted in FASL upregulation (Wei et al., 2011). This is consistent with the greater FASL expression observed in NOD.*D1Bax208*^{m1} mice, which had fewer GATA-3 binding sites than in NOD.*D1Bax208*^{m2} mice.

5.3.2 Polymorphism in FASL expression affects T1D

Studies using FAS deficient NOD$^{lpr/lpr}$ mice and FASL deficient NOD$^{gld/gld}$ mice, which demonstrated the defects in FAS/FASL system to provide complete protection against diabetes (Chervonsky et al., 1997; Su et al., 2000), led to the conclusion that FAS or FASL expression was detrimental to β-cell survival. Mice bearing homozygous *lpr* or *gld* mutations (defective *Fas* or *FasL* respectively), develop an age-dependent lymphoproliferative disorder, characterised by progressive accumulation of abnormal DN T cells (Watanabe-Fukunaga et al., 1992). It is possible that diabetes protection in these lpr and gld NOD mice could be an epiphenomenon associated with their ill health and grossly distorted immune system. The specific deletion of *Fas* on β-cells did not protect them from autoimmune destruction (Apostolou et al., 2003; Kim et al., 1999), suggesting limited role for FAS in β-cell destruction. Mice bearing *lpr* mutation compensated for FAS deficiency by overexpressing FASL on their T cells (Chu et al., 1995; Oura et al., 2013), and contrary to the assumption of FASL being detrimental in

autoimmune diabetes, it is possible that diabetes protection in these mice was an outcome of increased FASL expression on their cells. Chervonsky and colleagues found that NOD-RIP-FASL-31 mice with high levels of β-cell FASL expression resisted infiltrating T cells better when compared to NOD-RIP-FASL-24 mice whose β-cells expressed lower levels of FASL (Chervonsky et al., 1997). Paradoxically, in two other transgenic mouse models (RIP-CD95L^{hi} mice and NOD/FASL mice) high-level expression of CD95L was found to be detrimental to β-cells (Allison et al., 1997; Petrovsky et al., 2002). However, T or B cell infiltrates were remarkably absent in the islets of these mice, consistent with a FASL-mediated resistance against infiltrating cells. A mutation in the *Fasl* gene in NOD mice results in much lower activity than *Fasl* rom BALB/c mice, which are not prone to diabetes. This raises the likelihood that high FASL activity may have a protective effect on autoimmune diabetes. Consistent with the suggested protective role for FAS ligand, enhanced FASL expression in NOD.*D1Bax208*^{m1} mice was found to restrict their spontaneous diabetes incidence, when compared to NOD.*D1Bax208*^{m2} mice. NOD.*D1Bax208*^{m1} mice, which had increased expression of FASL had delayed diabetes onset, and the disease incidence was significantly reduced compared to NOD.*D1Bax208*^{m2} mice, which express lower levels of FASL. An intermediate disease incidence was noted in the control NOD/Lt mice, which express intermittent level of FASL. Even as the above results suggest a protective role for FAS death ligand in autoimmune diabetes, there existed a possibility that background genetic contamination, in either mouse line, could have contributed to

the differences in their disease development. A genome wide homogeneity screening revealed that NOD.*D1Bax208*^{m1} mice harboured a B6 DNA segment linked to the killer cell lectin-like receptor subfamily B member 1C (*Klrb1c*/CD161/NK1.1), between bases 91414637 to 117084506 on chromosome 6. Rest of the 685 single nucleotide polymorphisms (SNPs) genotyped from NOD.*D1Bax208*^{m1}, NOD.*D1Bax208*^{m2} and control NOD/Lt mice appeared to be identical. To exclude the possibility that differences in diabetes incidence in these two mouse lines, was an artefact of their respective genetic background, NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice were backcrossed with the parental NOD/Lt strain and the F_1 mice intercrossed to re-establish the mouse lines. Genetic homogeneity of the re-established NOD lines carrying the mutant alleles of *D1Bax208* was confirmed by typing 687 SNPs, none of which differed from the NOD/Lt parental strain. Diabetes incidence in the re-established NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mouse lines confirmed earlier findings, and in fact, the difference in survival rates between the two mouse lines appeared to be much more robust in the re-established lines (initial incidence of 76% vs. 50% in female and 53% vs. 21% in male, improved to 92% vs. 38% in female and 88% vs. 45% in male NOD.*D1Bax208*^{m1} mice and NOD.*D1Bax208*^{m2} mice respectively).This result suggested diabetes resistance in NOD.*D1Bax208*^{m1} mice to be an outcome exclusively attributable to its enhanced FASL expression. Kim and colleagues demonstrated that FASL overexpressing T cells could provide protection against diabetes by the elimination of FAS bearing autoreactive cells in an adoptive

transfer model (Kim et al., 2000), and more recently, chimeric FASL protein displayed on NOD diabetogenic splenocytes was demonstrated to be instrumental in delaying the progression and lowering the incidence of diabetes in an adoptive transfer model (Franke et al., 2007). Our data demonstrating delayed onset and reduced diabetes incidence in NOD line having enhanced FASL expression is consistent with the above findings, affirming a protective role for this apoptosis-inducing ligand in autoimmune diabetes. NOD mice which are prone to spontaneous diabetes have lower FASL expression and their T lymphocytes are less effective in triggering apoptosis of target cells compared to nondiabetes-prone BALB/c mice (Kayagaki et al., 1997). It was therefore likely that the increased prevalence of diabetes in NOD.*D1Bax208*^{m2} mice, which had lower FASL expression, was an outcome of defective elimination of autoreactive cells by cytotoxic T cells in the peripheral lymphocytic compartments in these mice. A flow cytometric examination of activated T cells in the spleens, livers and lymph nodes of NOD.*D1Bax208*^{m1} mice and NOD.*D1Bax208*^{m2} mice failed to identify any robust differences in their numbers or proportions after correction for multiple hypotheses testing. Even as the proportions of T cells in these two mouse lines appeared similar under unmanipulated situation, following stimulation by the mitogen Con A, T cells in the livers of NOD.*D1Bax208*^{m2} mice expanded significantly more than that in NOD.*D1Bax208*^{m1} mice (Figure 5.5 C). As activated murine T cells are sensitive to FAS/FASL-mediated cell death (Fortner and Budd, 2005; Ju et al., 1995), it was possible that, enhanced FASL expression on

NOD.*D1Bax208*^{m1} lymphocytes triggered the apoptosis of proliferating FAS^{+} T cells in their livers in a more efficient manner than in NOD.*D1Bax208*^{m2} mice. It has been reported that activated NKT cells upregulated FAS death ligand and its receptor and that they exhibited FASL mediated cytotoxicity (Arase et al., 1994; Chiba et al., 2008; Nowak et al., 2006; Wingender et al., 2010), and that they undergo rapid FASL-mediated apoptotic elimination in the livers of Con A treated mice (Takeda et al., 2000). Consistent with the above findings, hepatic NKT cells and NKT cell subsets of higher FASL expressing NOD.*D1Bax208*^{m1} mice contracted at a significantly greater rate post Con A injection than in NOD.*D1Bax208*^{m2} mice (Figure 5.6 A,B). This suggested that FASL expressing lymphocytes from NOD.*D1Bax208*^{m1} mice were more efficient in eliminating activated T and NKT cells than the lymphocytes from NOD.*D1Bax208*^{m2} mice.

Reciprocal adoptive transfer experiments in NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice revealed that accelerated diabetes was associated with low-FASL-expressing recipients (NOD.*D1Bax208*^{m2} recipients had a median survival time (MST) of 15 days as opposed to a MST of 67 days for NOD.*D1Bax208*^{m1} recipients, $p<0.0001$, life table analysis). This finding suggested a crucial role for FAS ligand expressed on nonhematopoietic tissues, in conferring a protective effect against autoimmune diabetes in NOD mice. While moderate leucocytic infiltration was noted in the islets of high-FASL-expressing NOD.*D1Bax208*^{m1} mice, florid lymphoid infiltrates were observed in the islets

NOD.*D1Bax208*^{m2} mice (Figure 5.12). Constitutive expression of FASL has been reported in human and NOD β-cells (Loweth et al., 1998; Redd et al., 2002) and FASL on islet cells have been reported to shield β-cells from immune attack (Signore et al., 1998). Yolcu and colleagues demonstrated FASL to be instrumental in extending the survival of allogeneic islets. Islets allografts co-transplanted with splenocytes displaying FASL chimeric with streptavidin (SA-FASL) prevented graft rejection and leucocytic infiltration (Yolcu et al., 2002). In a recent transplantation study, allogeneic islet grafts engineered to display SA-FASL on their surface overcame not only transplant rejection, but also autoreactive immunity in NOD mice (Yolcu et al., 2013). These findings suggest that FASL expression on islet cells could play a role in eliminating the infiltrating autoreactive cells in a FAS dependent manner (Figure 5.17). Moreover, islet-infiltrating effector cells are reported to be susceptible to FAS mediated apoptosis (Dharnidharka et al., 2002; Sainio-Pollanen et al., 1998). Consistent with this hypothesis, moderate level of islet infiltration was observed in high-FASL-expressing NOD.*D1Bax208*^{m1} mice as opposed to severe insulitis and extensive destruction in most of pancreatic islets of the low-FASL-expressing NOD.*D1Bax208*^{m2} mice. This finding is in accordance with the outcome of the reciprocal transfer experiment, which suggested a protective role for FASL expressed on nonhematopoietic tissues. FASL expression on endothelial cells was associated with VCAM-1 downregulation, which markedly reduced inflammatory cell migration (Sata and Walsh, 1998). VCAM- 1 blockade is known to delay the onset of diabetes in NOD mice, with a marked reduction in

lymphocytic infiltration (Baron et al., 1994; Kommajosyula et al., 2001). The moderate leucocytic infiltration observed in the pancreata of high FASL expressing NOD.*D1Bax208*^{m1} mice, compared to the massive infiltration seen in the pancreata of NOD.*D1Bax208*^{m2} mice could therefore be an outcome of islet-cell-FASL-mediated inhibition of chemokine secretion.

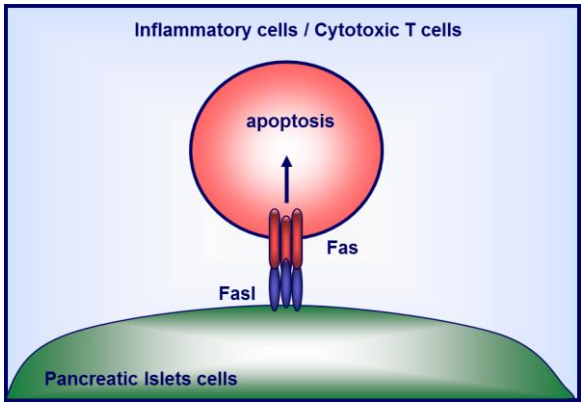


Figure 5.17 Proposed mechanism of FASL mediated islet-cell immune privilege.

Using bone marrow chimeras that expressed gld/gld mutation either in the hematopoietic or in the nonhematopoietic compartment of NOD mice Mohamood and colleagues illustrated that FASL levels in either of these compartments could affect autoimmune diabetes in NOD mice (Mohamood et al., 2007). Our reciprocal transfer experiments clearly demonstrated a protective role for FASL expressed on nonhematopoietic tissues, meantime it also suggested a minor intrinsic role for FASL in regulating the diabetogenicity of splenocytes. Mice receiving diabetogenic splenocytes from NOD.*D1Bax208*^{m2} mice had a MST of 43.5 days, whereas recipients of high-FASL-expressing NOD.*D1Bax208*^{m1} splenocytes had a MST of 77

days, indicating that higher FASL expression on splenocytes reduced their diabetogenicity. This finding is consistent with earlier reports that demonstrated FASL overexpression on T cells or Chimeric FASL protein displayed on diabetogenic cells inhibited diabetes in an adoptive transfer model (Franke et al., 2007; Kim et al., 2000). Moreover, T lymphocytes from higher-FASL-expressing BALB/c mice (non-diabetes prone) were more effective in triggering apoptosis of target cells compared to T cells from NOD mice (diabetes prone) that had lower levels of FASL expression (Kayagaki et al., 1997), indicating a role for lymphocytic FASL in regulating its diabetogenicity. In our study, increased FASL signalling (in NOD.*D1Bax208*^{m1} mice) was found to be associated with resistance to spontaneous diabetes, efficient elimination of lymphocytes and inhibition of cell proliferation after activation with Con-A. FASL, besides its role in delivering apoptotic signals through its receptor FAS, also functions as a negative modulator of certain immune responses, including, inhibition of T cell proliferation, cell cycle progression and IL-2 secretion, by means of FAS-FASL engagement or FASL reverse signalling (Bosque et al., 2008; Desbarats et al., 1998; Janssen et al., 2003; Luckerath et al., 2011). Significant upregulation of several genes implicated in lymphocyte proliferation and activation, including, *NFATc1*,25 *NF*-κ*B1*/*p50*, and Interferon Regulatory Factor 4 (*IRF4*) were noted in mice lacking the FASL intra-cellular domain (Luckerath et al., 2011). The deficiency of IRF4, expression of which is regulated by FASL signalling, is reported to have impaired the ability of T cells to secrete cytokines, including, IL-2 and IFN-γ (Mittrucker et al., 1997), suggesting

FASL reverse signalling mediated negative regulation. Contrary to our data that suggested FASL to be beneficial, Mohamood and colleagues suggested that inactivation or blockade of FASL expression was protective against autoimmune diabetes. They concluded based on adoptive transfer experiments using BM chimera that expressed gld/gld mutation and FASL neutralisation using anti-FASL antibody (MFL4) in NOD mice (Mohamood et al., 2007). However, in light of findings by Kayagaki and colleagues that lower FASL expressing T cells are less effective in triggering apoptosis of target cells (Kayagaki et al., 1997) and the above discussion on FASL-reverse-signalling mediated negative regulation, it appears that Mohamood and colleagues discounted the impaired diabetogenicity of T cells in their experimental models. Consistent with the finding that T cells had impaired ability to secrete cytokines, including, IL-2 and IFN-γ in mice lacking the FASL expression, our microarray analysis of islet transcripts identified the downregulation of *Socs1* (suppression of cytokine signalling) in the low FASL expressing NOD.*D1Bax208*^{m2} mice. SOCS1 is a strong inhibitor of JAK/STAT pathway and can attenuate the pathogenic effects of IFN-γ among others. SOCS1 is highly effective in protecting β-cells from perforin/cytokine mediated destruction. Overexpression of SOCS1 in insulin producing cells prevented IFN-γ-mediated β -cell death i*n vitro* (Cottet et al., 2001). *In vivo* NOD-RIP-SOCS1-Tg mice had reduced incidence of autoimmune diabetes, and the disease protection correlated with decreased IFN-γ-induced STAT-1 activation in β -cells and with reduced sensitivity of these cells to destruction by diabetogenic cells (Flodstrom-Tullberg

et al., 2003). NOD 8.3 mice with SOCS1 overexpression on their β-cells (NOD-RIP-SOCS1-Tg), did not proceed to diabetes (Chong et al., 2004). Expression of the highly potent chemokine IP-10 was inhibited in β-cells of RIP-GP/SOCS1^{+} mice, transgenic mice that expressed SOCS1 within the pancreatic β-cells (Barral et al., 2006). Results from the above studies suggest that SOCS1 is a key modulator of IFN-γ action and that it confers protection to islets at multiple levels. Thus, it was possible that FASL induction of SOCS1 in NOD.*D1Bax208*^{m1} mice negatively regulated IFN-γ-mediated β-cell destruction as well as IFN-γ-induced expression of chemokines. Our finding of low diabetes incidence and moderate islet infiltration in high-SOCS1-expressing NOD.*D1Bax208*^{m1} mice as opposed to the higher disease frequency and severe insulitis in low-SOCS1-expressing NOD.*D1Bax208*^{m2} mice is consistent with the β-cell protective effects of this protein described previously in NOD-RIP-SOCS1-Tg mice, NOD-RIP-SOCS1-Tg mice and RIP-GP/SOCS1^{+} mice. Taken together, the above findings suggest non-redundant roles for FASL expression in hematopoietic and non-hematopoietic compartments in the pathogenesis of autoimmune diabetes, and that some of the diabetogenic signals relied on interactions of FAS expressed on hematopoietic cells with FASL expressed on pancreatic cells and vice versa (Figure 5.18).

In summary, this study demonstrated that the pathogenic inflammatory responses leading to autoimmune diabetes are multifactorial, and depended on a complex combination of molecular, cellular and stochastic elements. Polymorphism at

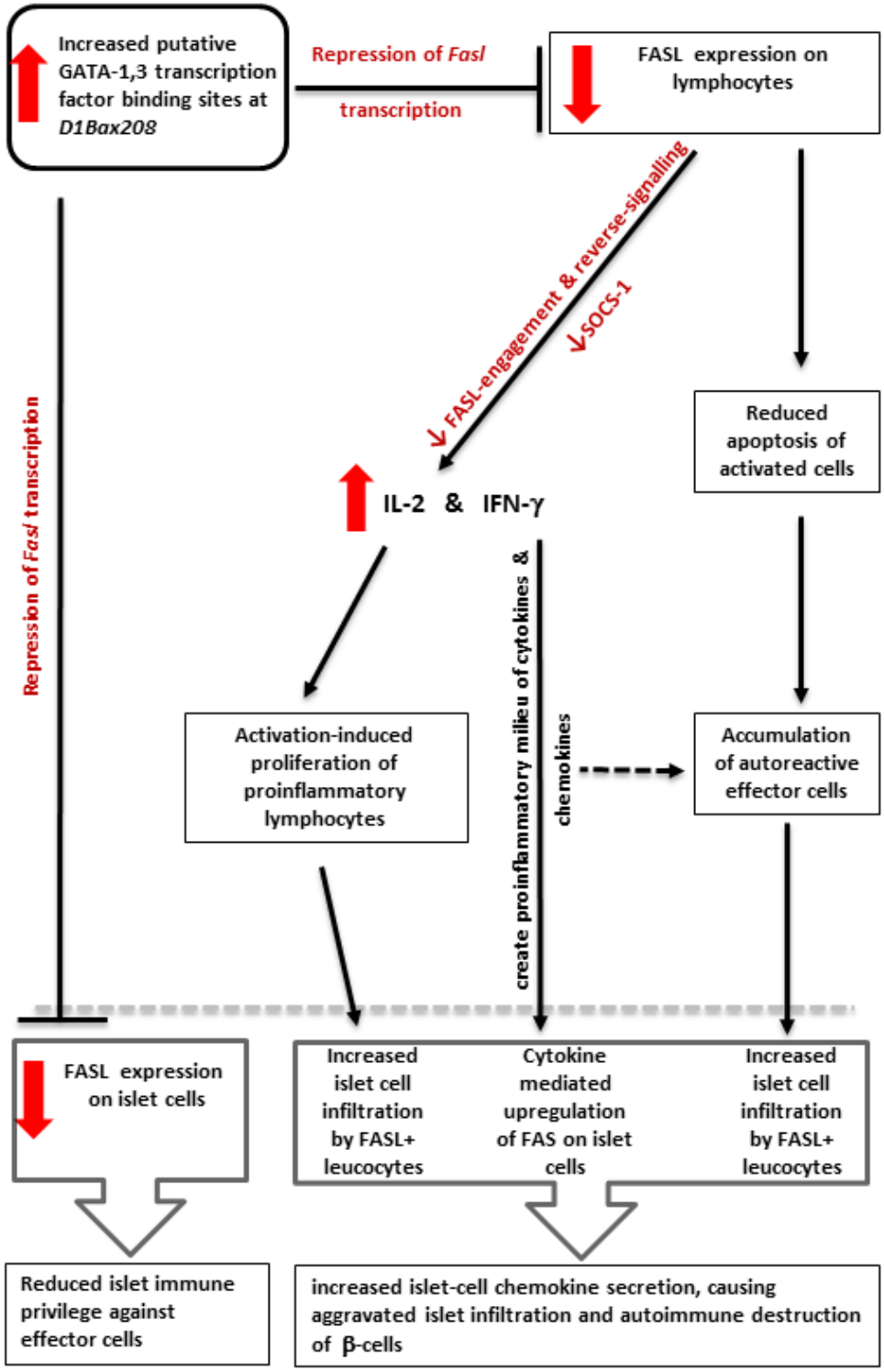


Figure 5.18 Proposed model illustrating how polymorphism in a *Fasl* simple sequence repeat affected T1D in NOD mice.

D1Bax208 microsatellite, which contained the binding sites for GATA transcription factor family, lying immediately 3' to *Fasl*, modified the level of transcription. Enhanced FASL expression was observed on lymphocytes from NOD.*D1Bax208*^{m1} mice, which had fewer GATA-3 transcription factor binding sites, consistent with a negative regulatory role of this transcription factor. Enhanced FASL expression in NOD.*D1Bax208*^{m1} mice appeared to play a crucial role in providing protection against autoimmunity as these mice were found to have significantly delayed onset and reduced incidence of spontaneous autoimmune diabetes compared to NOD.*D1Bax208*^{m2} mice, which expressed lower levels of FASL. The outcome of reciprocal transfer experiment, suggested a crucial role for FASL expressed on nonhematopoietic tissues, in providing protection against islet-reactive effector cells. Results of microarray analysis of islet transcripts suggest FASL-signalling-mediated inhibition of SOCS1 secretion. SOCS1 can attenuate the pathogenic effects of cytokines, especially IFN-γ. It was also likely that FASL expressed by the islet-cells themselves provided immune privilege against effector-cell infiltration. Activated lymphocytes in higher-FASL-expressing NOD.*D1Bax208*^{m1} mice were found to contract at a higher rate than lymphocytes in NOD.*D1Bax208*^{m2} mice. It was therefore likely that increased level of FASL expression in NOD.*D1Bax208*^{m1} mice, besides inducing increased apoptosis of activated autoreactive cells, also induced higher levels of FASL reverse-signalling, dampening cell proliferation, and inhibiting the secretion of proinflammatory cytokines in these mice; all these amounting to reduced diabetogenicity of lymphocytes.

.....

CHAPTER 6

.....

GENE ENVIRONMENT INTERACTIONS IN NOD AUTOIMMUNITY

.....

CHAPTER 6.1

.....

Environmental agent mediated modulation of NOD diabetes

6.1.1 Introduction

T1D is an organ specific autoimmune disorder, possibly precipitated by environmental agent(s) acting in the context of a predisposing genetic background. Exposure to microbes or microbial products initially triggers an immune response mediated by the innate immune system. Such exposure may initiate, enhance or even suppress autoimmunity both in humans and in animal models of T1D (illustrated in Tables 2.3 and 2.4), suggesting a potential role for innate immunity in autoimmune disease predisposition. The NOD mouse strain is a well-characterised animal model of autoimmune diabetes, the disease incidence of which is greatly affected by environmental factors.

Microbial agents have long been used (in the form of vaccines or adjuvants) to modulate immune responses. NOD mice treated with BCG (live attenuated *M.bovis*) or CFA (killed *M. tuberculosis*) are protected from autoimmune diabetes (Harada et al., 1990; Qin et al., 1993; Sadelain et al., 1990). IFN-γ appeared to mediate the protection in the above cases as neither BCG nor CFA was successful in preventing diabetes in IFN-γ deficient NOD.*Ifng*$^{-/-}$ mice (Serreze et al., 2000). BCG (mycobacteria) recognition by innate immune cells via pattern recognition receptors known as Toll like receptors (TLRs) resulted in rapid activation of the innate immune system (Heldwein et al., 2003; von Meyenn et al., 2006). IFN-γ production is the key for protection against mycobacterial infection (Cooper et al.,

1993; Flynn et al., 1993; Hogan et al., 2001), and NK cells, another constituent of innate immune system, are crucial in the initial response against mycobacterial infections (de la Barrera et al., 2004; Wolfe et al., 1976). BCG, an attenuated mycobacterial preparation, is known to elicit the expansion of NK cell-numbers and also to induce the upregulation of IFN-γ secretion by NK cells (Lee et al., 2008; Yang et al., 1995). Thus, an intriguing association exist between environmental agents (BCG), its recognition receptors (TLRs), innate lymphocytes (NK cells) and proinflammatory cytokines (IFN-γ).

This study hypothesizes that the association between microbes and the innate immune system in NOD mice could influence the strains expression of autoimmunity. Using NOD mouse lines bearing targeted mutations for *Ifn-γ* (NOD.*Ifng*$^{-/-}$) and the β chain of *Ifn-γ* receptor (NOD.*Ifngr2*$^{-/-}$) and congenic NOD.NK1.1 mice (NOD.*Nkrp1*^{b}), this study aims at characterizing the underlying mechanism(s) and specific roles of various components of innate immune system, in BCG mediated modulation of autoimmunity in NOD mice.

6.1.2 Results

6.1.2.1 Spontaneous diabetes incidence in NOD/Lt mice.

NOD mice were originally derived from a single diabetic female detected during the breeding of a cataract-prone strain. They vary in the incidence of diabetes between colonies, even those that are genetically homogeneous. It is therefore advisable that the incidence of diabetes in each NOD colony be monitored and used as a reference. In order to determine the spontaneous diabetes incidence of NOD mice colony housed at our SPF facility, random blood glucose from 37 female and 22 male NOD/Lt mice were monitored from 8 weeks to 32 weeks of age (Figure 6.1 A). More than 70% female mice developed the disease, significantly higher than that of around 25% in males (27/37 (73%) vs. 6/22 (27.3%); $p<0.0005$, life table analysis; $p<0.0007$, Fisher's exact test).

6.1.2.2 BCG provides protection against diabetes in NOD mice.

A low to high dose-titration was performed to determine the effects of BCG (*Mycobacterium bovis)* on diabetes incidence in NOD mice. While mid to high doses of BCG (0.25, 0.5 and 1.0mg) delayed the onset of diabetes and provided significant protection against the disease ($p<0.01$, life table analysis), mice that received 0.1mg BCG were only marginally protected compared to the saline group (Figure 6.1 B).

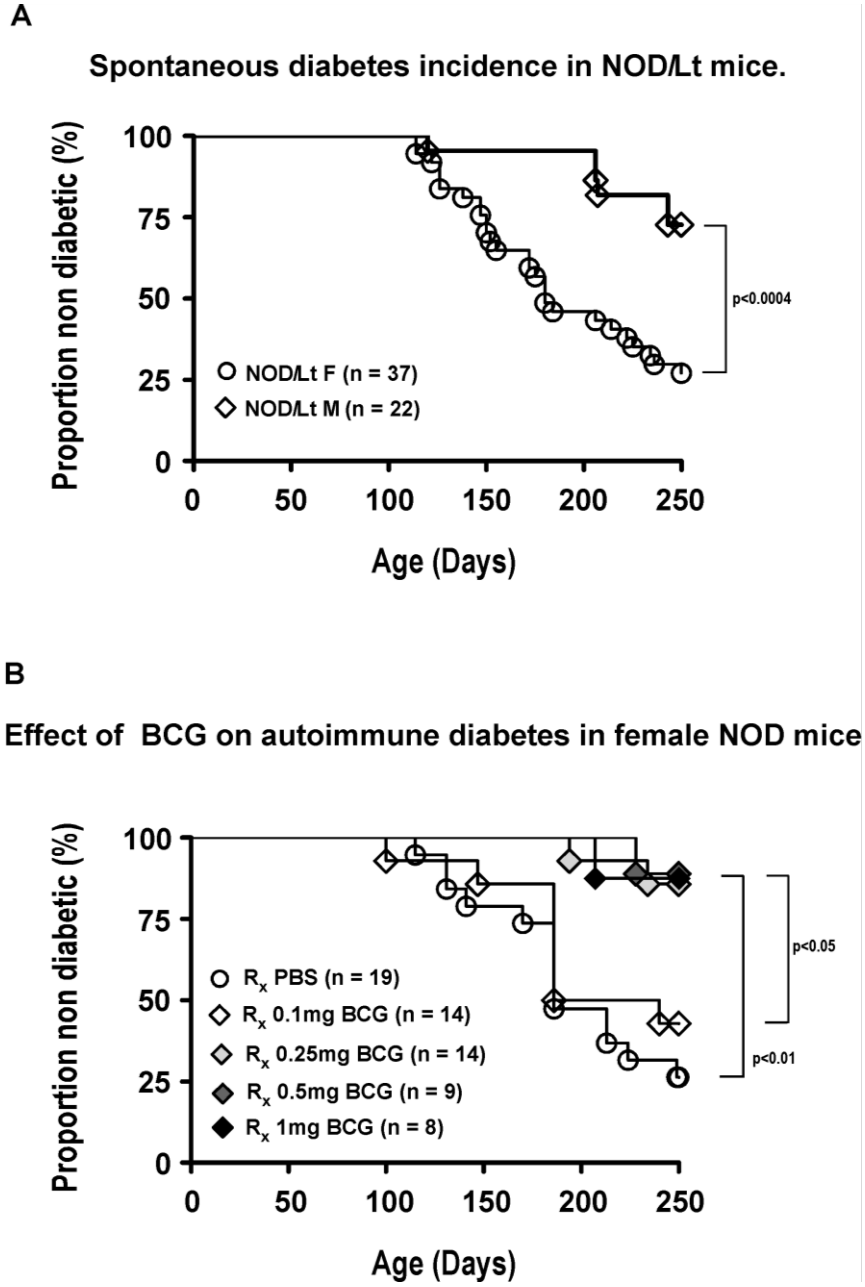


Figure 6.1 Spontaneous diabetes in NOD/Lt mice. Female mice had a higher incidence compared to male NOD mice (A). Diabetes incidence in female NOD mice immunized at 8 week of age with a series of M.*bovis* preparations (B). Higher doses of BCG (0.25mg, 0.5mg and 1mg) delayed the onset of diabetes and provided significant protection while 0.1mg dose had no effect when compared to saline group (significance calculated by Fisher's exact test).

However, the disease resistance conferred by 0.25mg was comparable to that offered by 0.5mg and 1mg groups (Table 6.1), and hence was the preferred dose used in all further experiments.

BCG Rx dose	**% diabetic**	**p value (vs. control, Fisher's exact test)**
None (saline)	73.7 (n=19)	-
0.1mg	57.1 (n=14)	0.4587
0.25mg	14.3 (n=14)	0.0013
0.5mg	11.1 (n=9)	0.0036
1.0mg	12.5 9n=8)	0.0085

Table 6.1 The lowest dose of BCG that offered significant protection against **autoimmune diabetes** in NOD mice was 0.25mg.

6.1.2.3 Diabetes can be adoptively transferred into irradiated recipients.

Various concentrations of diabetogenic splenocyte preparations (5 million, 10 million, 15 million or 20 million cells) were transferred into irradiated female NOD recipients. All the recipients of 20 and 15 million cells developed diabetes within 8 weeks, however only 1 recipient of 10 million cells and none from the group that received 5 million cells developed the disease during the 12 week study (Figure 6.2 A, $p<0.04$ (20 million vs. 5 million group, life table analysis)). Splenocyte preparations with 15 or 20 million cells were used in all further adoptive transfer experiments.

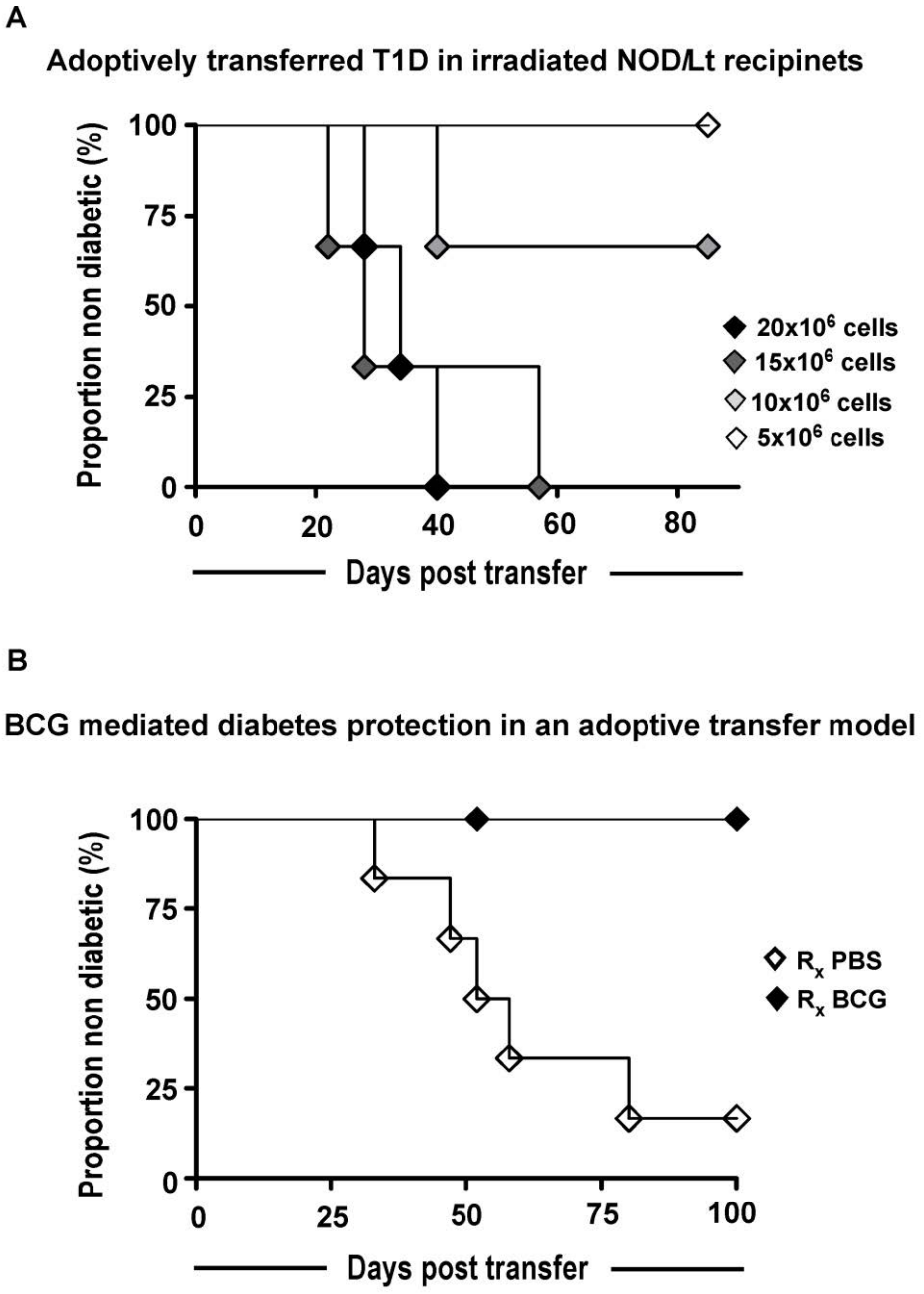


Figure 6.2 Titration of diabetogenicity of splenocytes transferred from diabetic NOD mice into irradiated female NOD/Lt recipients (A, n= 3 in each group). 0.25mg BCG protected adoptive transfer of diabetes in NOD.Scid mice (B, n= 6 in either groups, $p<0.02$, Fisher's exact test).

6.1.2.4 BCG protects against disease in the adoptive transfer model.

Having known the protective nature of BCG against spontaneously occurring autoimmune diabetes in NOD mice, it was interesting to explore the effect of a similar treatment in an adoptively transferred model of the disease. Splenocytes from diabetic NOD mice were transferred into twelve NOD.*scid* recipients, of which 6 were treated with 0.25mg BCG and the remaining with saline. None of the 6 NOD.*scid* recipients immunised with BCG developed the disease while 5 out of 6 (83.3%) saline administered mice developed diabetes during the 100 day study period (Figure 6.2B; $p<0.006$, life table analysis, $p<0.02$, Fisher's exact test), confirming a role for BCG in inhibiting the efficacy of diabetogenic cells.

6.1.2.5 BCG induced an elevation in systemic TNF and IFN-γ levels.

BCG induces cytokine production in mice. Levels of IL-2, IL-4, IL-5, TNF and IFN-γ in NOD/Lt serum were quantified using a cytometric bead array at 30 minutes, 90 minutes, 180 minutes and 4 weeks post 1mg BCG instillation. A temporal elevation in the levels of TNF and IFN-γ was noted (Figure 6.3). While IFN-γ levels gradually increased over the 4 weeks post BCG treatment, an immediate spike in TNF production (even as early as 30 minutes post treatment) was observed. Levels of other cytokines measured, (IL-2, IL-4 and IL-5) were below the detection limits (not shown).

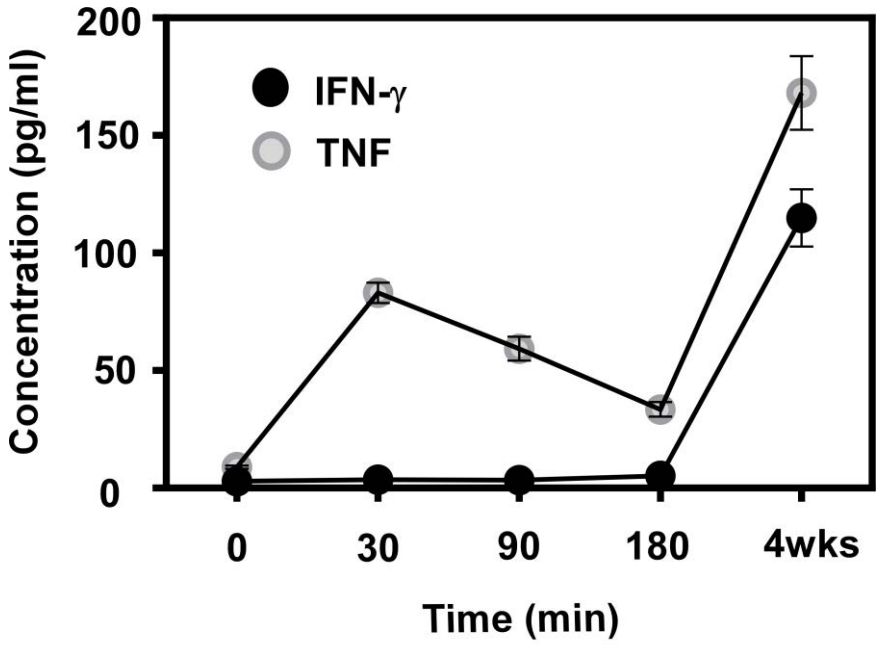


Figure 6.3 Quantitation of serum cytokines in response to 1mg BCG (ImmuCyst) treatment in NOD/Lt mice. Levels of IFN-γ and TNF in treated mice depicted in the box plot. Levels of IL-2, IL-4 and IL-5 were below detection limits at these time points.

6.1.2.6 IFN-γ deficiency did not alter spontaneous diabetes in female NOD mice.

Previous studies considered IFN-γ to be essential for the development of autoimmune diabetes (von Herrath and Oldstone, 1997; Wang et al., 1997). The blood glucose levels of female NOD.*Ifng*$^{-/-}$ mice and their WT counterparts were assessed at regular intervals for 35 weeks to determine the role played by this cytokine in diabetes development. The results however revealed a similar disease incidence in either group (Figure 6.4 A). However, female NOD mice with the functionally inactivated gene encoding the β-chain of the *Ifn-γ* receptor (NOD.*Ifngr2*$^{-/-}$), had a significantly lower incidence (NOD 27/37(73%) vs. NOD.*Ifngr2*$^{-/-}$ 11/31 (35.5 %) $p<0.002$; NOD.*Ifng*$^{-/-}$ 14/22 (63.6%) vs. NOD.*Ifngr2*$^{-/-}$ 11/31 (35.5 %) $p<0.02$, Fisher's exact test). Early disease onset and higher diabetes incidence was observed in male NOD.*Ifng*$^{-/-}$ mice when compared to WT or NOD.*Ifngr2*$^{-/-}$ mice (mean survival time of 211 days, 238 days and 242 days respectively; $p=0.0814$, life table analysis, Figure 6.4 B). The lower incidence of spontaneous diabetes in male NOD mice compared to their female littermates is a standard feature of the model. Albeit subdued, the male NOD.*Ifngr2*$^{-/-}$ mice, similar to their female counterpart, had late onset and reduced diabetes incidence than WT NOD/Lt mice. It was likely that the naturally low penetrance of diabetes in male NOD mice could have potentially masked further evidence of inhibition of this trait in the NOD.*Ifngr2* deficient environment.

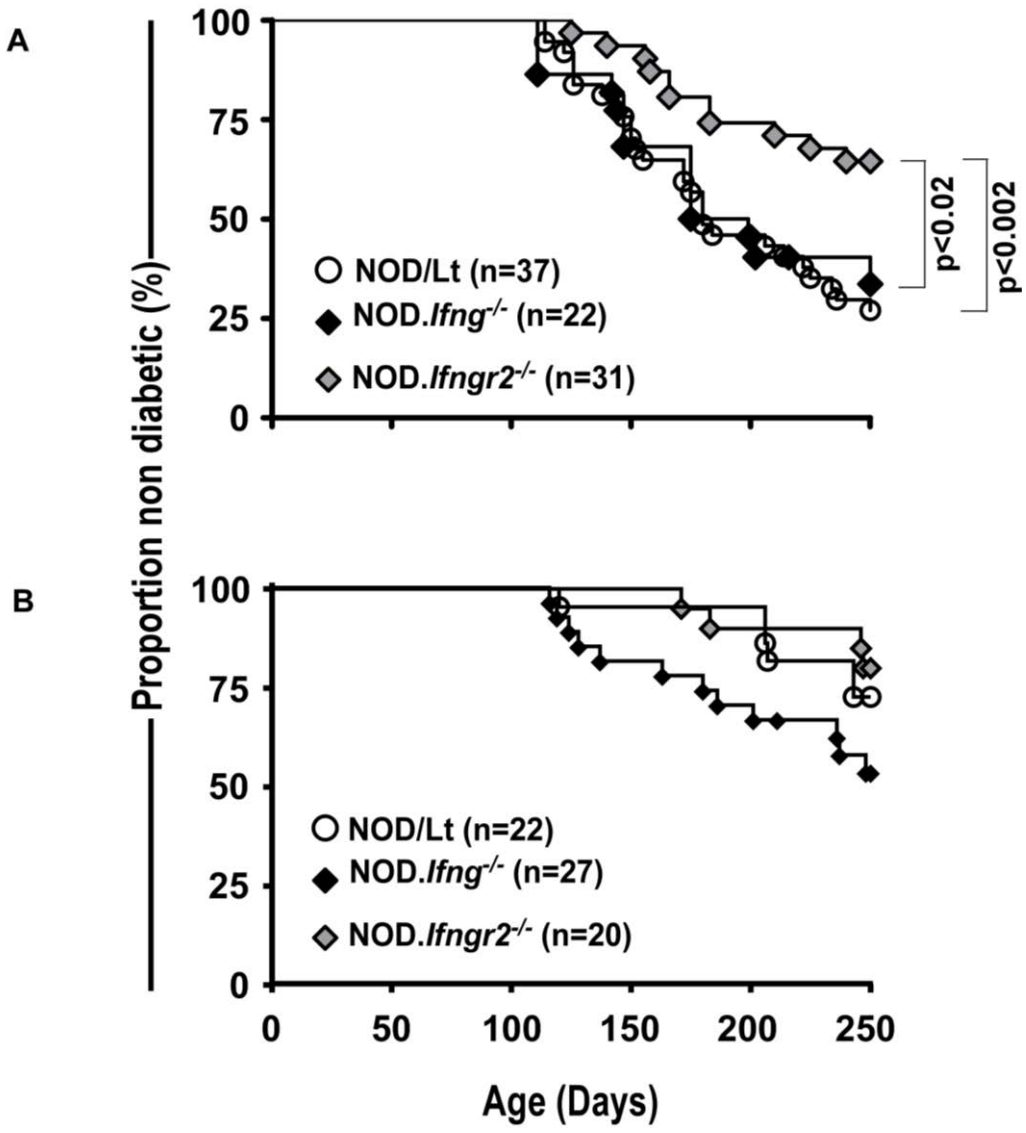


Figure 6.4 Spontaneous diabetes development in female (A) and male (B) NOD.*Ifng*$^{-/-}$, NOD.*Ifngr2*$^{-/-}$ and the wild type NOD/Lt mice. While incidence in female NOD.*Ifng*$^{-/-}$ was similar to the WT, there was a significant reduction in disease incidence of NOD.*Ifngr2*$^{-/-}$ mice ($p<0.002$, Fisher's exact test). The incidence in the male cohorts was not different.

6.1.2.7 IFN-γ deficiency nullified the protection offered by a low dose mycobacterial immunisation in NOD mice.

The immunisation dose of BCG that prevented diabetes development in NOD/Lt mice (0.25mg) failed to evoke a similar response in IFN-γ deficient NOD mice. BCG treated WT NOD mice were mostly protected from diabetes with the disease incidence around 20% (4/19), compared to around 80% (12/15) in the saline group (Figure 6.5A and B). Interestingly, BCG administered NOD.*Ifng*$^{-/-}$ mice had 15% more diabetics (89.5% (17/19)) compared to the saline treated group (73.7% (14/19)). Meanwhile the incidence in NOD.*Ifngr2*$^{-/-}$ mice was similar irrespective of the treatment (46.7 % (7/15) and 44.4 % (4/9) respectively, Figures 6.5A and B). Even as the 0.25mg BCG treatment offered no protection against diabetes in the IFN-γ and the IFN-γR2 deficient mice, immunisation with 1.0mg BCG conferred a near total protection in all the 3 mouse lines (Figure 6.5C). The above findings suggest that while IFN-γ is crucial for BCG mediated diabetes-protection in NOD mice, at higher doses IFN-γ independent diabetes protective mechanism(s) might come into action.

6.1.2.8 BCG offered no protection against adoptively transferred disease from diabetic NOD.*Ifng*$^{-/-}$ mice or NOD.*Ifngr2*$^{-/-}$ mice.

Splenocytes from recently diabetic NOD/Lt, NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice were intravenously transferred into NOD.*scid* mice. Cells from IFN-γ deficient mice transferred diabetes in an accelerated manner compared to splenocytes from

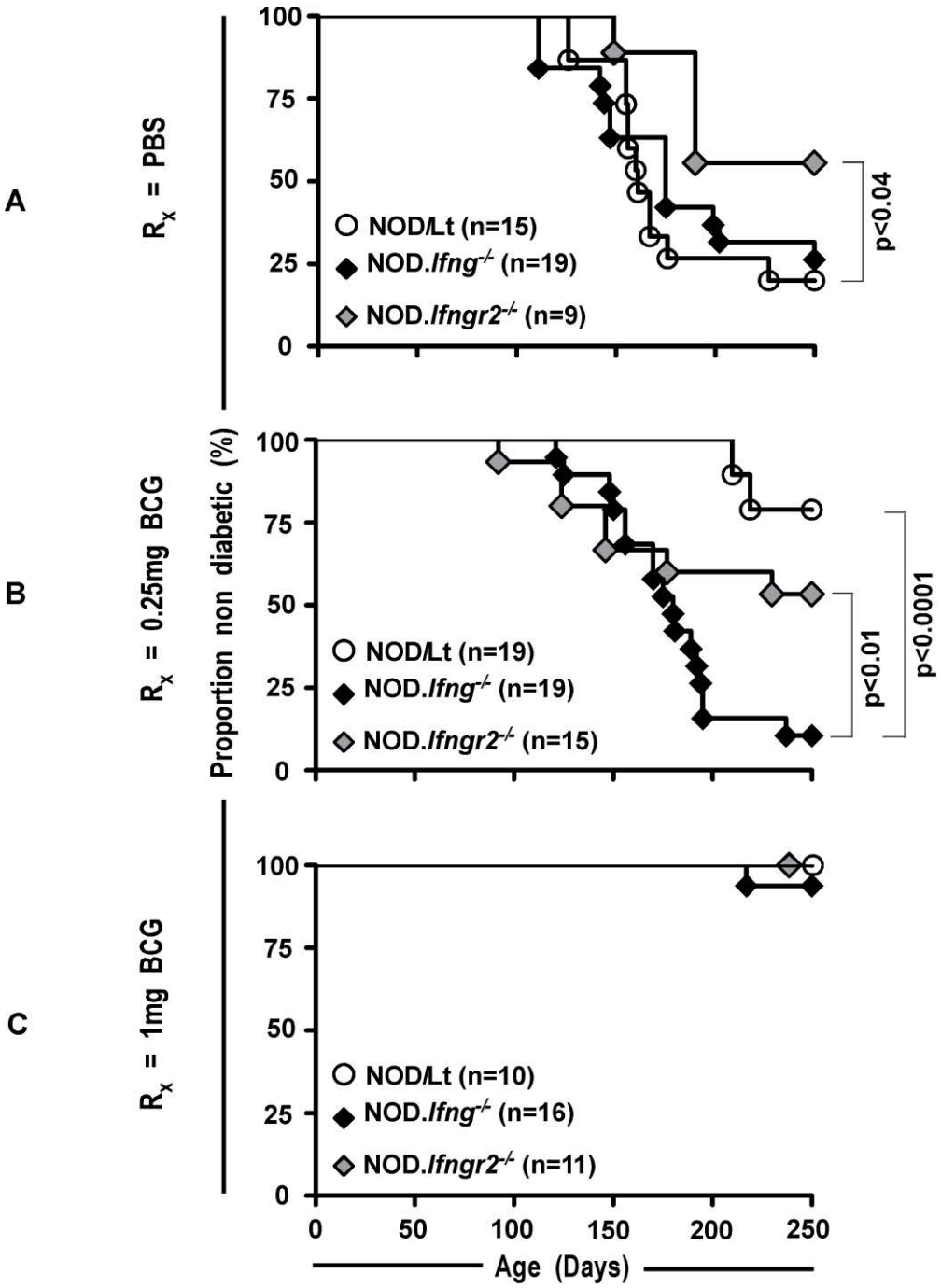


Figure 6.5 Survival of female NOD/Lt, NOD.*Ifng*$^{-/-}$, NOD.*Ifngr2*$^{-/-}$ mice treated with 0.25mg and 1mg dose of BCG, compared with saline controls. P values by Fisher's exact test.

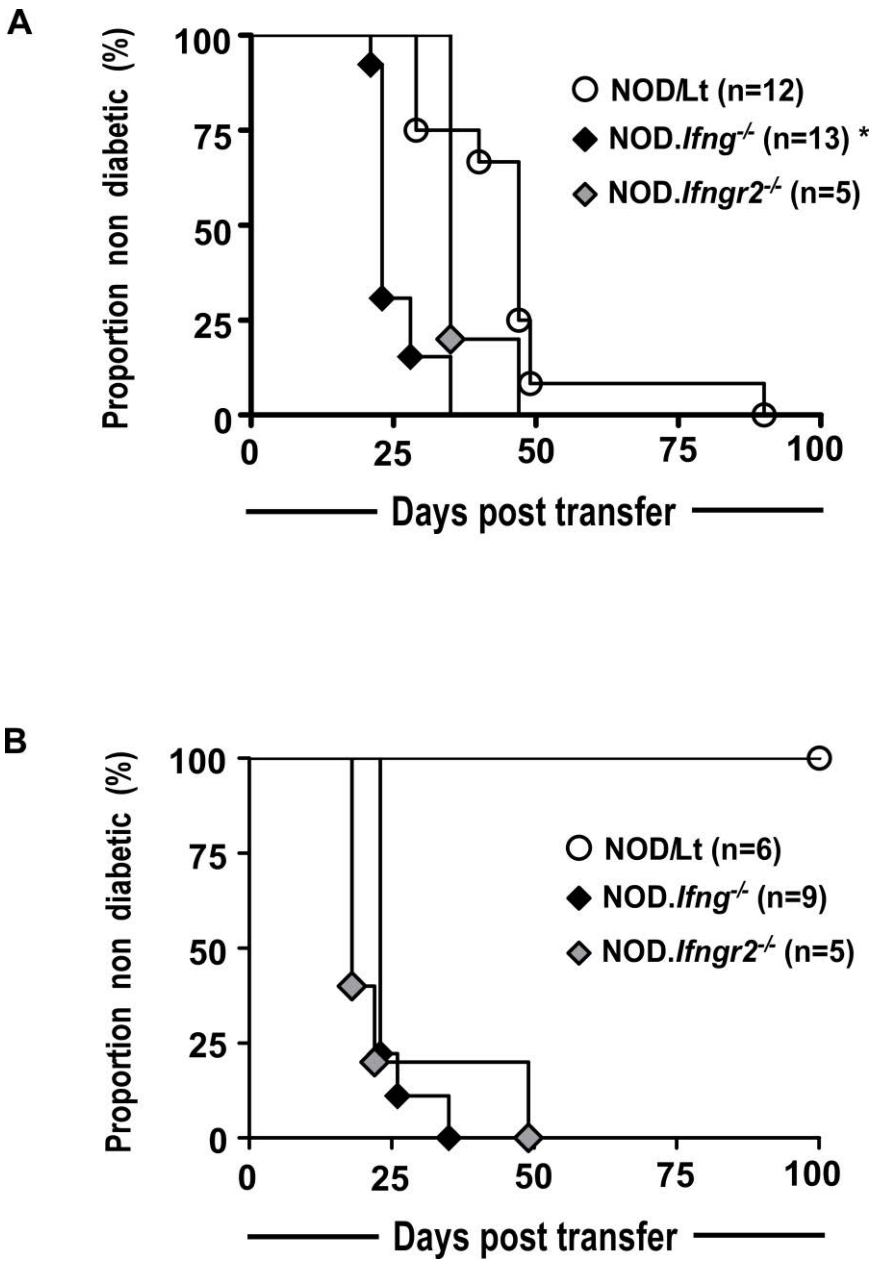


Figure 6. 6 Adoptive transfer of diabetogenic splenocytes into female NOD.*scid* mice from diabetic NOD/Lt, NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice (A). 0.25mg BCG offered total protection against adoptively transferred diabetes from NOD/Lt, while it failed to protect the transfer of disease effected by cells from IFN-γ and IFN-γR2 deficient NOD mice (B). * $p<0.0001$ vs. NOD/Lt and $p<0.003$ vs. NOD.*Ifngr2*$^{-/-}$, life table analysis.

WT mice (p<0.0001, life table analysis) or from mice deficient in the IFN-γ receptor 2 (p<0.003, life table analysis, Figure 6.6A). Immunisation of NOD.*scid* recipients with 0.25mg BCG 7 days post transfer did not confer any protection against disease transferred from IFN-γ or IFN-γR2 deficient mice, as opposed to the recipients of WT cells, which were totally protected against the disease (p<0.0001, life table analysis, Figure 6.6B).

6.1.2.9 Higher proportions of Foxp3+ T cells observed in INF-γ and IFN-γ receptor β chain deficient NOD mice.

The regulatory T cell population, defined by the expression of forkhead box P3 (Foxp3) protein, was detected by intracellular staining and flowcytometry. An increased proportion of Foxp3+ T cells was observed in the spleens of both the gene knockout mutant strains when compared to wild type NOD mice (p<0.008 in both cases, Mann Whitney U test, Figure. 6.7). Similarly higher proportions of Tregs were seen in the livers of NOD.*Ifng*$^{-/-}$ mice (p<0.008, Mann Whitney U test, Figure. 6.7). FoxP3^{+} T cell proportions in lymph nodes in all three groups of mice appeared similar (Figure. 6.7). Proportions and numbers of Tregs did not differ between the thymi of these mice (data not shown). Taken together these results indicate that the genetic ablation of IFN-γ or IFN-γR2 did not diminish the proportions of Tregs in NOD mice. On the contrary, the higher proportions of peripheral Tregs in these mice compared to WT mice suggest an inhibitory role for IFN-γ in the induction of this immunoregulatory cell population.

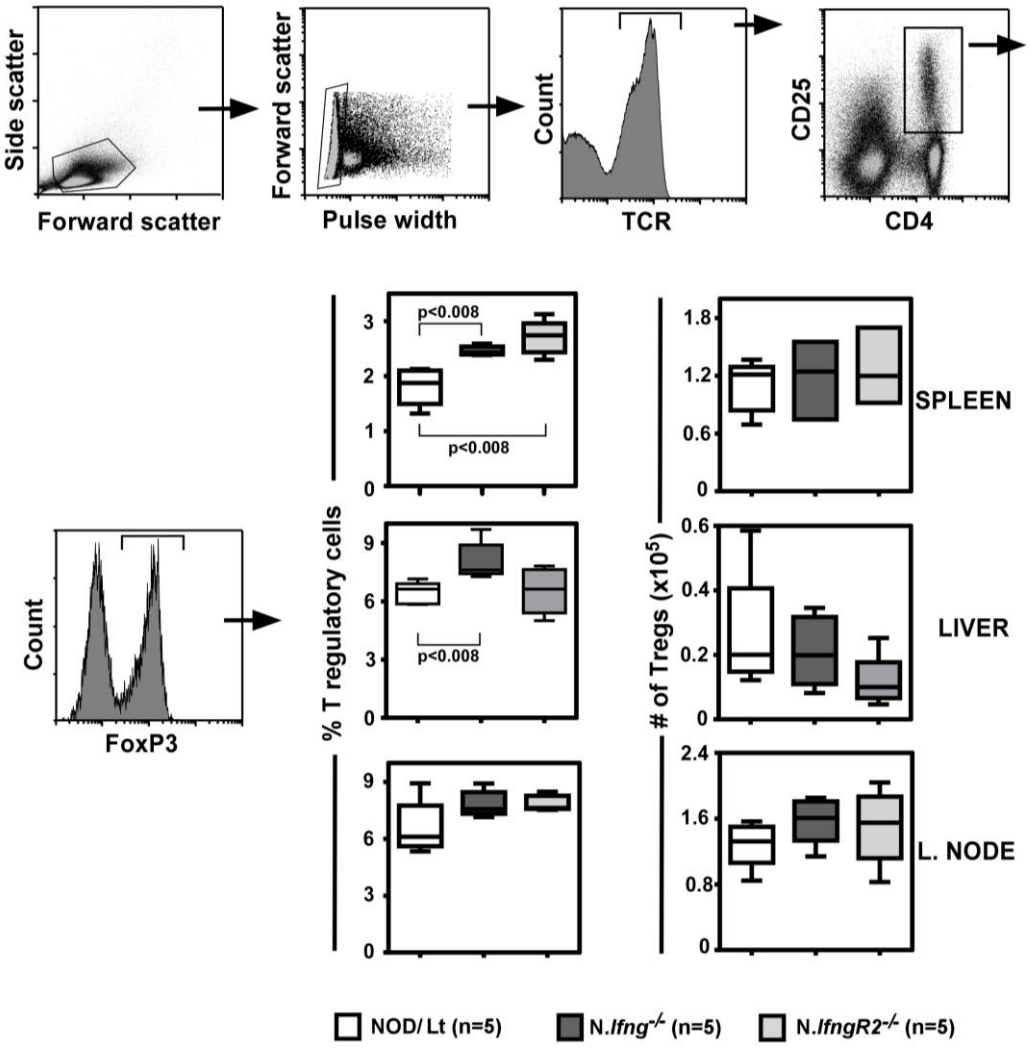


Figure 6.7 Higher proportions of regulatory T cells in the secondary lymphoid organs of IFN-γ and IFN-γR2 deficient NOD mice. Absolute numbers of Tregs however, were similar in these mice. 5 mice in each group. p value by Mann-Whitney U statistics

6.1.2.10 Higher proportions of NKT cells observed in INF-γ and IFN-γ receptor β chain deficient NOD mice post BCG treatment.

The numbers and proportions of NKT cell populations were analysed in untreated wild type NOD, NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice. No differences were observed except for a reduction of NKT cell numbers in NOD.*Ifngr2*$^{-/-}$spleens when compared to wild type NOD mice ($p<0.008$, Mann Whitney U test, Figure 6.8 A). In a separate experiment, NOD/Lt, NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice were treated with BCG and their NKT cell numbers compared one-week post treatment. An increased splenic NKT cell proportion was observed in NOD.*Ifngr2*$^{-/-}$ mice when compared to both NOD/Lt and NOD.*Ifng*$^{-/-}$ mice ($p<0.003$ and $p<0.009$ respectively, Mann Whitney U test, Figure 6.8 B). Higher numbers of NKT cells were observed in livers of NOD.*Ifngr2*$^{-/-}$ mice when compared to NOD/Lt and NOD.*Ifng*$^{-/-}$ mice ($p<0.009$ and $p<0.02$ respectively, Mann Whitney U test, Figure 6.8 B). Spleen NKT cell numbers and proportions of NKT cells in livers of NOD.*Ifngr2*$^{-/-}$ mice followed a similar trend, but did not attain statistical significance. These findings indicate that absence of IFN-γ or IFN-γR2 did not affect the proportions of NKT cells in NOD mice.

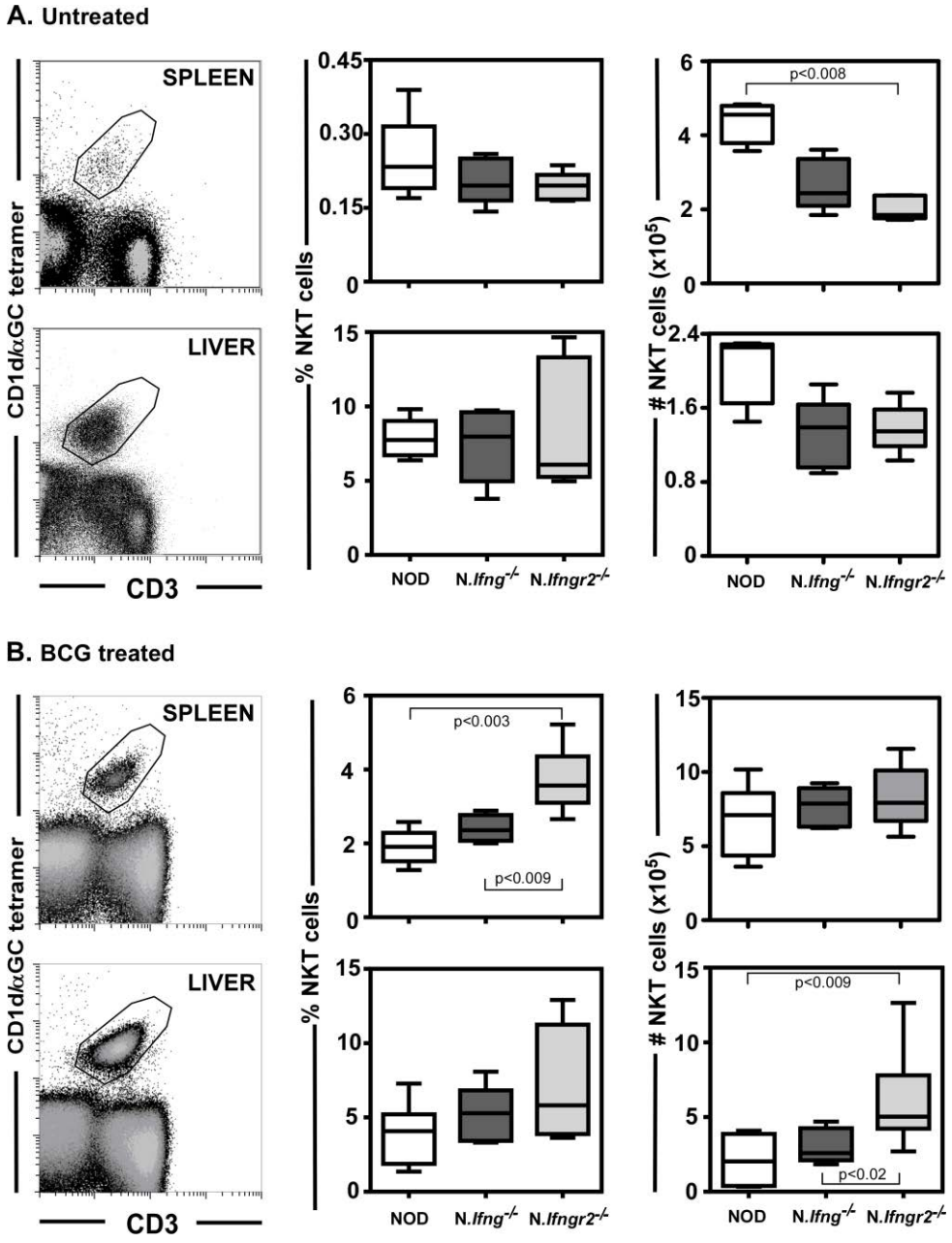
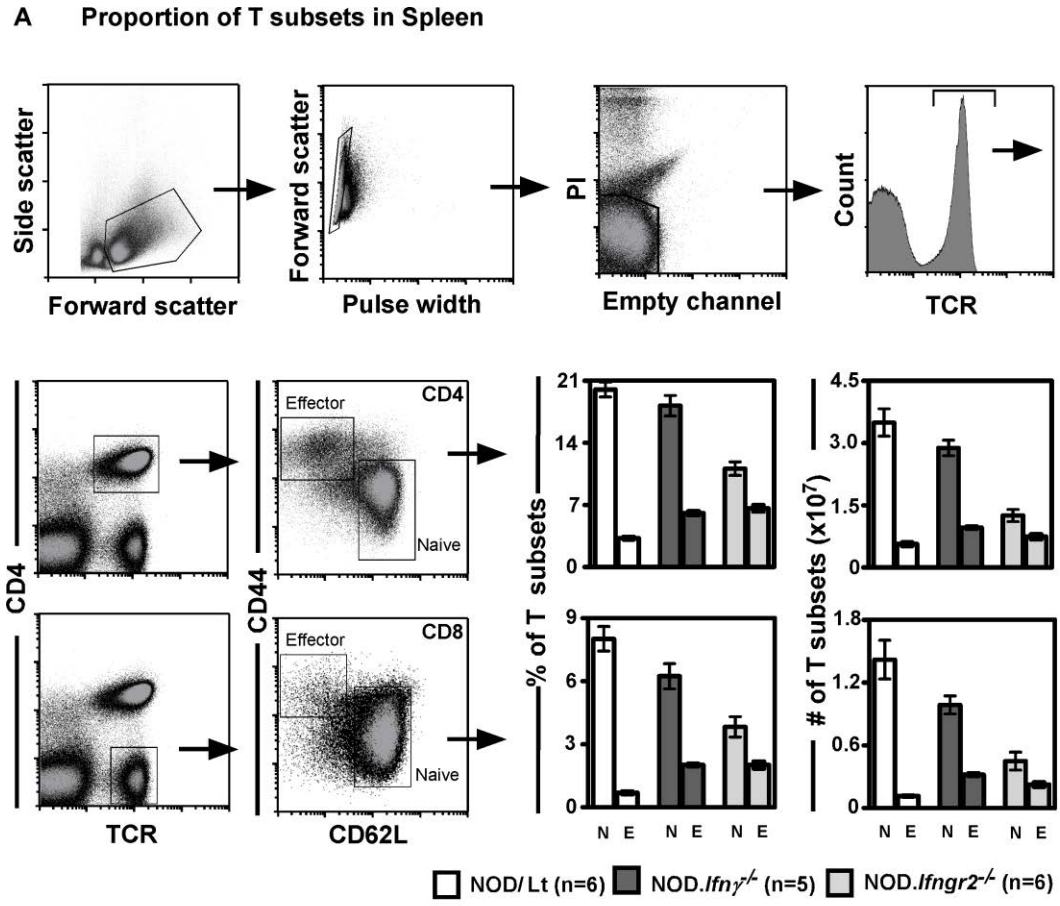


Figure 6.8 Proportions and numbers of NKT cells in the peripheral organs of untreated (A) and BCG treated (B) NOD mice and its IFN-γ and IFN-γR2 deficient counterparts. Representative dot plots showing the NKT cell gating scheme in experiments consisting 5 mice per each group in A and NOD/Lt =5, NOD.*Ifng*$^{-/-}$ =6 and NOD.*Ifngr2*$^{-/-}$ =5 in B.

6.1.2.11 Higher proportions of effector T cells observed in INF-γ and IFN-γ receptor β chain deficient NOD mice post BCG treatment.

The proportions of naïve ($CD44^{low}CD62L^{+}$) and effector ($CD44^{+}CD62L^{-}$) phenotype $CD4^{+}$ and $CD8^{+}$ T cells in the spleens and livers of pre-diabetic female NOD mice carrying the mutant alleles of *Ifng* and *Ifngr2* were compared to that of parental NOD/Lt mice one week post 0.25mg BCG administration. Flow cytometric analyses revealed significantly higher proportions and numbers of activated T cells in the secondary lymphoid organs of NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice (summarised in Figure 6.9 A, Table 6.2 (spleen) and Figure 6.10 A and Table 6.3 (liver)). The ratio of naïve: effector T cells in the wild type NOD/Lt mice were significantly higher compared to that of NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice (Figure 6.9 B (spleen), 6.10 B (liver), Table 6.4, the differences were true after multiple hypothesis testing by the Bonferroni method). Higher proportions and numbers of activated T cells represented by cell surface markers CD4, CD122, CD132, and CD25 were observed in the lymph nodes of NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice when compared to that of parental NOD/Lt mice (data not shown). These findings are suggestive of an IFN-γ-mediated suppression or elimination of effector T cells in BCG treated NOD mice.



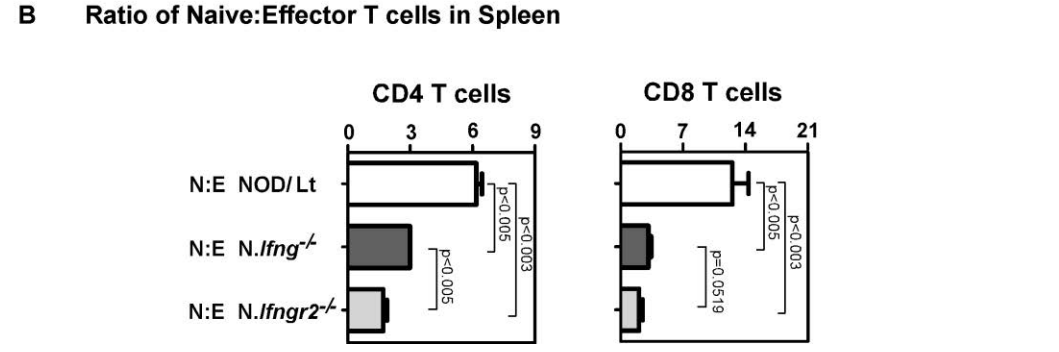


Figure 6. 9 T cell subsets of spleen. Proportions and numbers of naive and effector $CD4^+$ and $CD8^+$ T cells (A) and naive: effector T cell ratio (B) in the spleens of IFN-γ and IFN-γR2 deficient NOD mice along with wild type NOD/Lt mice. N = Naive, E = Effector, N:E = Naive to Effector ratio. p value by Mann-Whitney U statistics.

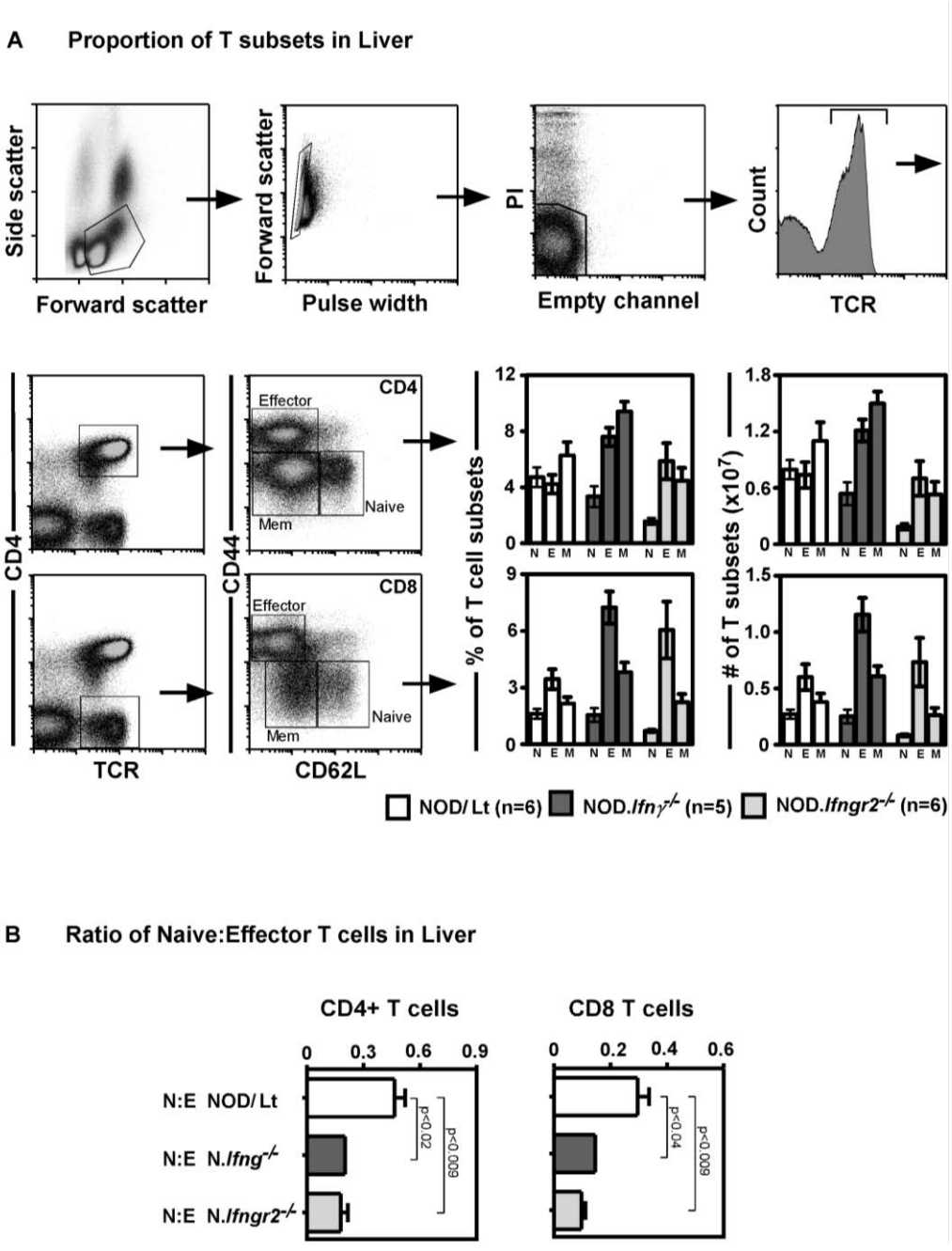


Figure 6. 10 T cell subsets of liver. Proportions and numbers of naive and effector $CD4^+$ and $CD8^+$ T cells (A) and naive: effector T cell ratio (B) in the spleens of IFN-γ and IFN-γR2 deficient NOD mice along with wild type NOD/Lt mice. N = Naive, E = Effector, N:E = Naive to Effector ratio. p value by Mann-Whitney U statistics.

Comparison	T cells	% Naive	% Effector	# Naïve	# Effector
NOD vs. NOD.*Ifng*$^{-/-}$	CD4	ns	<0.005	ns	<0.005
	CD8	ns	<0.005	ns	<0.005
NOD vs. NOD.*Ifngr2*$^{-/-}$	CD4	<0.003	<0.003	<0.003	<0.02
	CD8	<0.003	<0.003	<0.003	<0.005
N.*Ifng*$^{-/-}$ vs. N.*Ifngr2*$^{-/-}$	CD4	<0.005	ns	<0.005	<0.04
	CD8	<0.04	ns	<0.009	<0.005

Table 6. 2 Summary of statistical comparisons of the proportions (%) and numbers (#) of naïve and effector CD4$^+$ and CD8$^+$ T cells in the spleen. Significance by Mann Whitney U test.

Comparison	T cells	% Naive	% Effector	% Memory	# Naïve	# Effector	# Memory
NOD vs. NOD.*Ifng*$^{-/-}$	CD4	ns	<0.009	ns	ns	<0.04	ns
	CD8	ns	<0.009	<0.04	ns	<0.04	ns
NOD vs. NOD.*Ifngr2*$^{-/-}$	CD4	<0.003	ns	ns	<0.003	ns	ns
	CD8	<0.02	ns	ns	<0.003	ns	ns
N.*Ifng*$^{-/-}$ vs. N.*Ifngr2*$^{-/-}$	CD4	ns	ns	<0.005	ns	ns	<0.005
	CD8	ns	ns	ns	ns	ns	<0.02

Table 6.3 Summary of statistical comparisons of the proportions (%) and numbers (#) of naïve and effector CD4$^+$ and CD8$^+$ T cells in the liver. Significance by Mann Whitney U test.

Comparison	T cells	Spleen	Liver
NOD vs. NOD.*Ifng*$^{-/-}$	CD4	<.0005	<0.02
	CD8	<.0005	<0.04
NOD vs. NOD.*Ifngr2*$^{-/-}$	CD4	<.0003	<0.009
	CD8	<.0003	<0.009
N.*Ifng*$^{-/-}$ vs. N.*Ifngr2*$^{-/-}$	CD4	<.0005	ns
	CD8	<.0519	ns

Table 6.4 Summary of statistical comparisons of naïve to effector T cell ratios in the secondary lymphoid organs of NOD/Lt, NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice. Significance by Mann Whitney U test.

6.1.2.12 BCG induced IL-17 levels were lower in NOD.*Ifngr2*$^{-/-}$ mice.

IL-17 is a proinflammatory cytokine known to synergise with both IFN-γ and TNF in the induction of autoimmune disease (Bending et al., 2009; Martin-Orozco et al., 2009). However, a recent study suggests a protective role for CFA-induced IL-17 in NOD diabetes (Nikoopour et al., 2010). We compared IL-17 and TNF-α production in IFN-γ and IFN-γR2 deficient NOD mice with that of wild type NOD mice. Intracellular flowcytometry on whole splenocytes 24 hours post 0.25mg BCG immunisation revealed higher numbers and proportions of IL-17 producing cells in wild type NOD mice when compared to NOD.*Ifngr2*$^{-/-}$ mice ($p<0.003$ and $p< 0.009$ respectively, Mann Whitney U test, Figure 6.11A). The absolute numbers and proportions of IL-17 producing splenocytes in NOD.*Ifng*$^{-/-}$

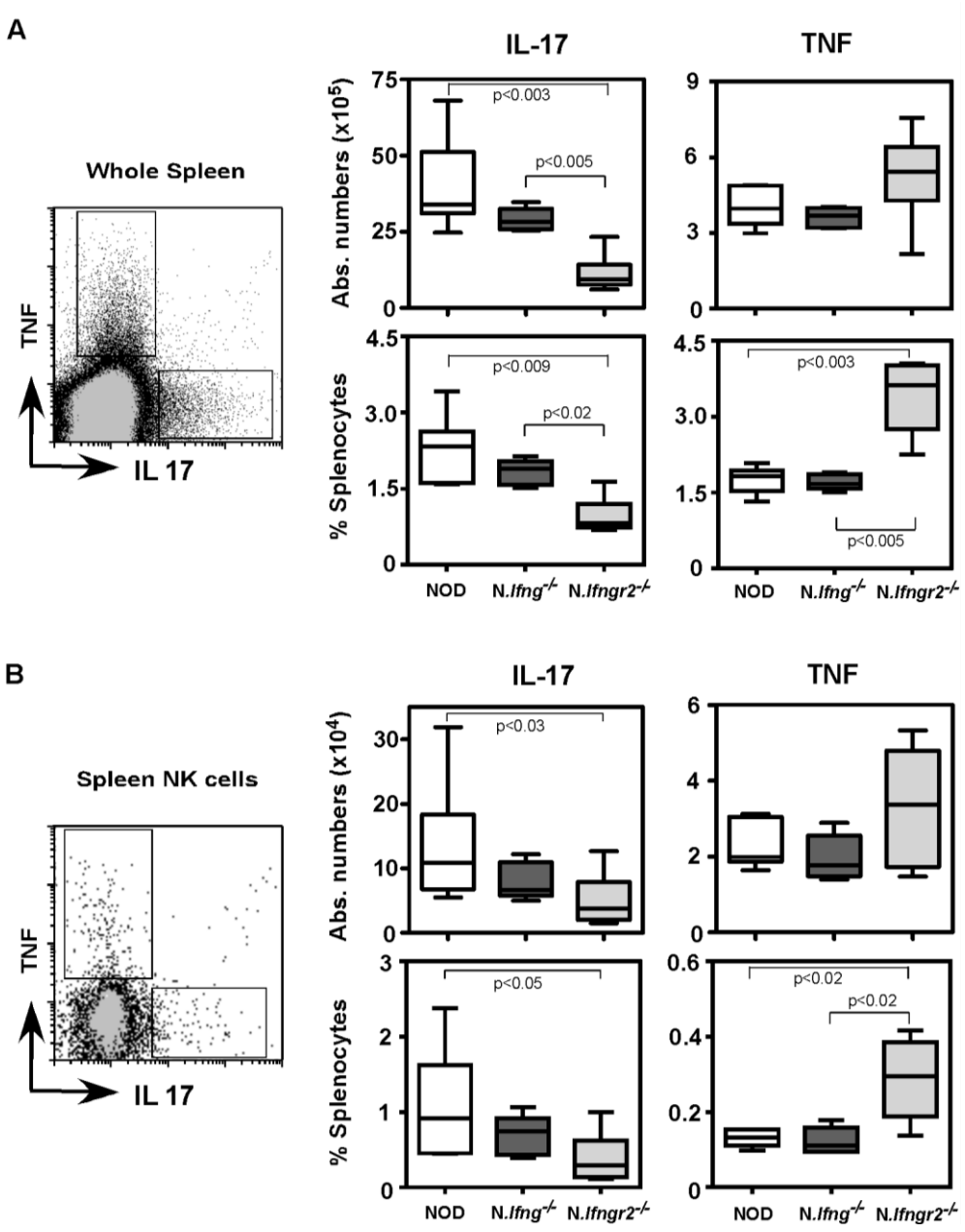


Figure 6.11 **T**NF and IL-17 expressing splenocytes of IFN-γ and IFN-γR2 deficient NOD mice, compared to WT NOD mice. IFN-γR2 deficient NOD mice had higher numbers of TNF expressing cells, but significantly lower IL-17 production, especially evident in NK cells. NOD.*Ifng*$^{-/-}$ (n=5), NOD.*Ifngr2*$^{-/-}$ (n=6) NOD/Lt (n=6); p values by Mann Whitney U test.

mice were higher when compared to NOD.*Ifngr2*$^{-/-}$ mice ($p<0.005$ and $p<0.02$ respectively, Mann Whitney U test, Figure 6.11A). Numbers of TNF producing splenocytes were similar in all three groups, but its proportions were higher in NOD.*Ifngr2*$^{-/-}$ mice compared to the other two groups ($p<0.003$ (NOD/Lt Vs. NOD.*Ifngr2*$^{-/-}$) and $p<0.005$ (NOD.*Ifng*$^{-/-}$ Vs. NOD.*Ifngr2*$^{-/-}$), Mann Whitney U test, Figure 6.11A). IL-17 and TNF secretion by NK cells was determined by gating on splenic NK cells defined by the pan-NK cell marker DX5. In a trend similar to that of whole splenocytes, wild type NOD mice had higher numbers and proportions of IL-17 producing NK cells compared to NOD.*Ifngr2*$^{-/-}$ mice ($p<0.03$ and $p<0.05$ respectively, Mann Whitney U test, Figure 6.11B). Numbers of NK cells that secreted TNF were similar in all three groups, however proportions of NK cells that produced TNF were higher in the spleens of NOD.*Ifngr2*$^{-/-}$ mice compared to both NOD/Lt and NOD.*Ifng*$^{-/-}$ mice ($p<0.02$ in both cases, Mann Whitney U test, Figure 6.11B). From the above findings, it appears that BCG-induced IL-17 upregulation in NOD mice is IFN-γ-dependent, and NK cells are the likely producers.

6.1.2.13 BCG-induced IL-17 levels in an adoptive transfer model.

To further understand the role of BCG-induced IL-17 responses in diabetes protection, diabetogenic splenocytes from NOD/Lt, NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice were transferred into three separate groups of NOD.*scid* mice. All the NOD.*scid* recipients were then immunised with 0.25 mg BCG. While 60% (3/5) of

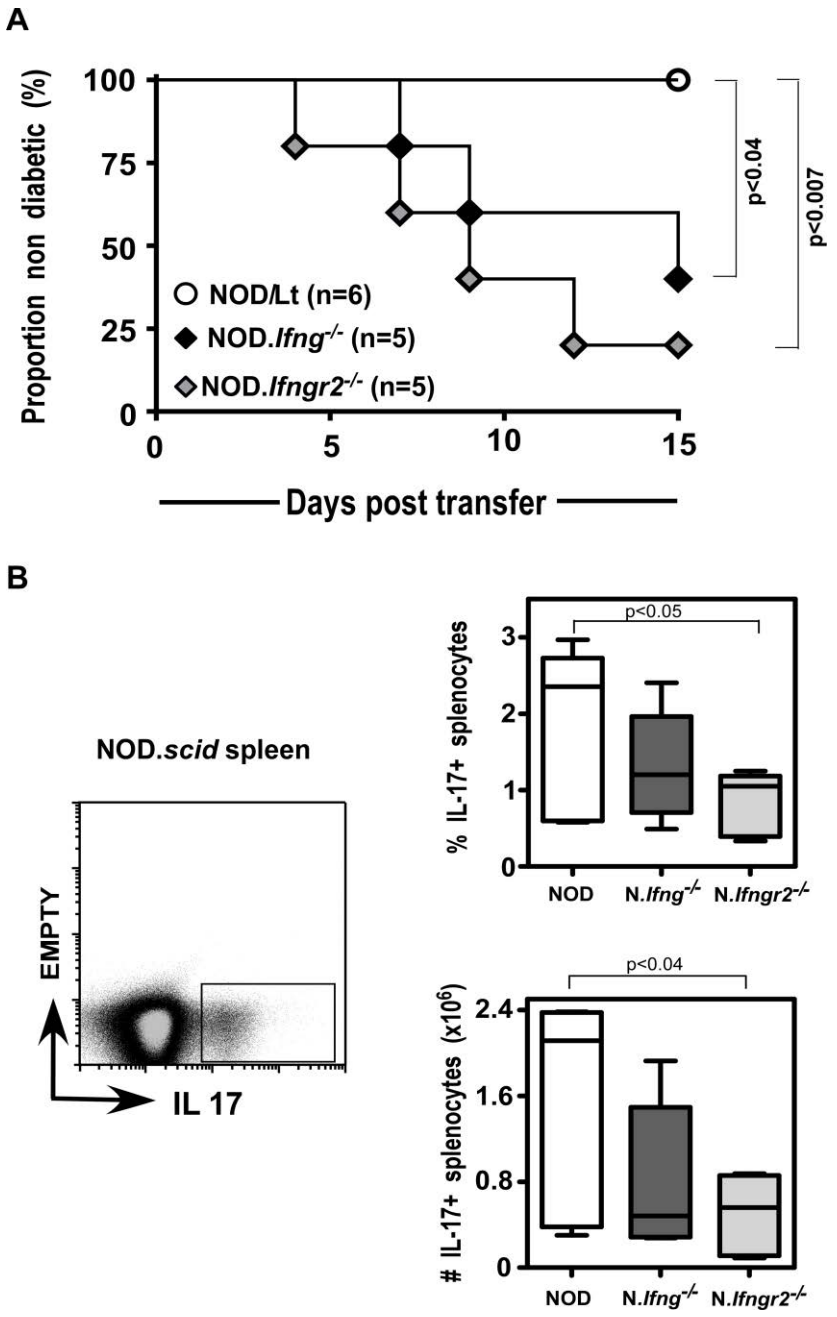


Figure 6.12 Life table analysis of 0.25mg BCG immunised NOD.*scid* recipients of diabetogenic splenocytes from wild type NOD/Lt and IFN-γ and IFN-γR2 deficient NOD mice (A). Intracellular IL-17 levels in the above NOD.*scid* recipients 2 weeks post transfer (B). p values by unprotected Fisher's LSD test.

recipients of IFN-γ-deficient splenocytes and 80% (4/5) of recipients of IFN-γR2-deficient cells developed diabetes within 2 weeks of transfer, none of the recipients of wild type splenocytes (0/6) developed the disease in the 2 week study period ($p<0.04$ (NOD/Lt Vs. NOD.*Ifng*$^{-/-}$) and $p<0.007$ (NOD/Lt Vs. NOD.*Ifngr2*$^{-/-}$), life table analysis, Figure 6.12 A). Intracellular flowcytometry on NOD.*scid* splenocytes at the end of the study revealed that proportions and numbers of IL-17 secreting cells were lower in the recipients of IFNgR2- deficient cells than in the recipients of wild type cells ($p=0.0445$ and 0.0302, unprotected Fisher's LSD test, Figure 6.12 B). Although not statistically significant, a similar trend of lower IL-17 levels was observed in NOD.*scid* recipients of IFN-γ-deficient cells as well (Figure 6.12 B). These findings are indicative of an association between IFN-γ-signalling and IL-17 production in BCG-mediated immune response in NOD mice.

6.1.2.14 NK cell depletion did not alter BCG mediated diabetes protection in a syngeneic adoptive transfer model.

Role of NK cells in diabetes development as well as its influence on BCG-mediated protection against the disease was determined by adoptively transferring diabetogenic splenocytes which were magnetically depleted of cells expressing the NK cell marker (NK1.1), from NOD.*Nkrp1*^{b} mice (Figure 6.13A) into NOD.*scid* mice. Seven days later, recipients were immunised with 0.25mg BCG or

were saline treated. NK cell depletion did not diminish the BCG-mediated protection

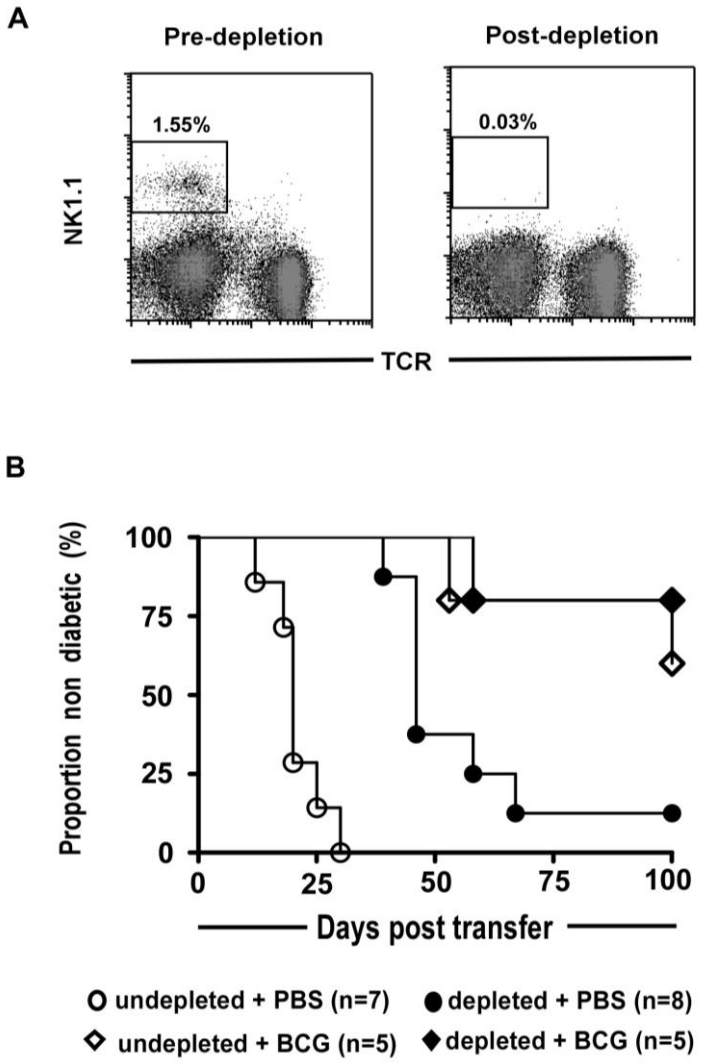


Figure 6.13 Diabetogenicity of NK cell depleted splenocytes. Significant reduction of NK1.1 expressing cells post depletion confirmed by flowcytometry (A). BCG mediated protection of diabetes in an adoptive transfer model. Undepleted or NK cell-depleted diabetogenic NOD.*Nkrp1*^{b} splenocytes transferred into NOD.*scid* recipients, immunised with BCG or saline 7 days post transfer (B). Comparison of survival curves summarised in Table 6.6.

against diabetes observed in the recipients of undepleted cells (Figure 6.13B). Absence of NK cells however caused a significant delay in the transfer of diabetes in saline treated recipients. While 85% of NOD.*scid* recipients of undepleted splenocytes developed diabetes within 3 weeks of transfer, none of the recipients of NK cell-deficient transfers went diabetic in the same period (p=0.0001, life table analysis, Figure 6.13B), suggesting a role for these cells in precipitating NOD diabetes. Outcomes of the transfer experiments and comparisons of survival curves are summarised in tables 6.5 and 6.6 respectively.

NK cells	Treatment	Diabetes development
Undepleted	Saline	7/7
Depleted	Saline	7/8
Undepleted	BCG	2/5
Depleted	BCG	1/5

Table 6.5 Development of diabetes in BCG or saline treated NOD.*scid* mice adoptively transferred with diabetogenic splenocytes from NOD.*Nkrp1*^{b} mice, before or after NK cell depletion.

		Rx	PBS	PBS	BCG	BCG
		NK Cells	+	-	+	-
NK Cells	Rx					
+	PBS			0.0001	0.001	0.001
-	PBS		0.0001		<0.05	<0.02
+	BCG		0.001	<0.05		NS
-	BCG		0.001	<0.02	NS	

Table 6.6 Summary of survival curve comparisons. Significance by Mantel-Cox test (log-rank test).

6.1.2.15 Characterisation of diabetes susceptibility in NOD.*Tlr2*$^{-/-}$ mice.

Among known agonists for mammalian Toll like receptor (TLR) proteins are several products derived from mycobacteria that have been found to activate cells in a TLR2-dependent manner. It is therefore likely that TLRs, especially TLR2, might affect spontaneous diabetes as well as influence BCG mediated modulation of NOD autoimmunity. To test this hypothesis, blood glucose levels of NOD.*Tlr2*$^{-/-}$ mice and their heterozygous and WT littermates were assessed at regular intervals to 35 weeks age. Comparison of the incidence of spontaneous diabetes indicated that TLR2 deficiency conferred a significant protection against the development of the disease. Life table analysis revealed that while 80% (24/30) of NOD.*Tlr2*$^{+/+}$ mice became diabetic, only less than 50% (10/22) of NOD.*Tlr2*$^{-/-}$ littermates developed the disease during the same observation period (Figure 6.14A; $p<0.01$, Mantel-Cox test; $p<0.02$, Fisher's exact test), suggesting a significant contribution of TLR2 in the development of spontaneous autoimmune diabetes. Immunisation of NOD.*Tlr2*$^{-/-}$ mice with 0.25mg BCG offered significant protection from autoimmune diabetes when compared to saline treated control mice. While none of the 13 BCG treated TLR 2 deficient mice became diabetic, 43% of its saline treated littermates developed the disease in the 35 week study period (Figure 6.14 B; $p<0.02$, Mantel-Cox test; $p<0.02$, Fisher's exact test).

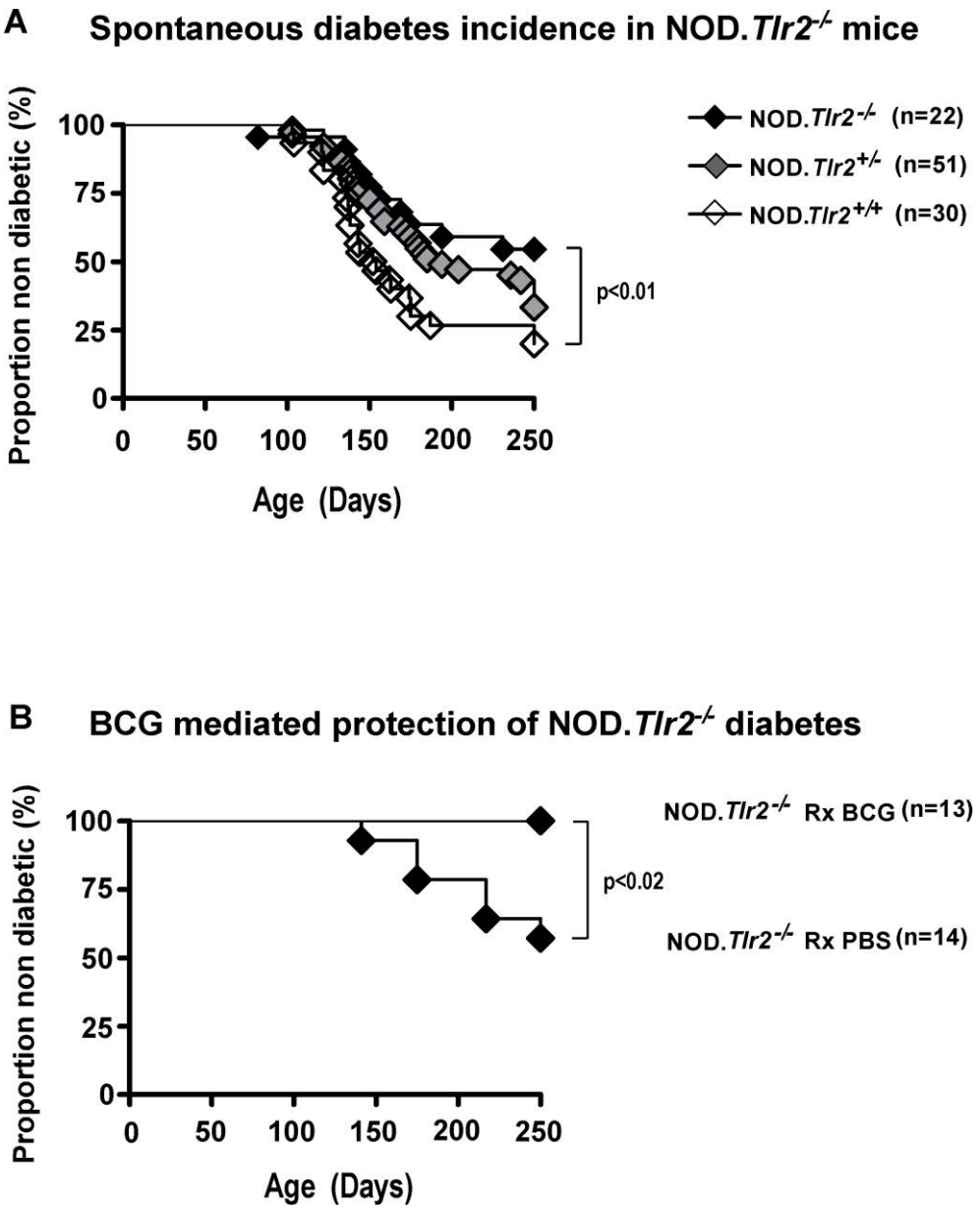


Figure 6.14 Cumulative incidence of spontaneous diabetes in litter mate female NOD.*Tlr2*$^{+/+}$, NOD.*Tlr2*$^{+/-}$ and NOD.*Tlr2*$^{-/-}$ mice (A). BCG mediated protection against spontaneous diabetes in NOD.*Tlr2*$^{-/-}$ mice (B)

.....

CHAPTER 6.2

.....

Environmental agent mediated precipitation of autoimmunity in NOD mice

6.2.1 Introduction

NOD mice spontaneously develop autoimmune diabetes, but immunization with BCG prevents the onset of this disease. Even as BCG provides protection against diabetes in NOD mice, it mediates the precipitation of an autoimmune condition similar to SLE (Baxter et al., 1994). While *M. bovis*- mediated IFN-γ secretion appeared to play a key role in diabetes protection in NOD mice (Serreze et al., 2001), an elevated expression of this cytokine was reported in SLE patients (Harigai et al., 2008). Over production of IFN-γ was found to be required for lupus-like disease in MRL-*lpr* mice (Balomenos et al., 1998). The fact that a single BCG injection prevented diabetes but induced lupus, and that IFN-γ appeared to be crucially associated with these two outcomes, suggested a possible pleiotropic role for this cytokine in NOD autoimmunity. Excessive IFN-γ production in response to BCG could have possibly caused the switch between the two phenotypic expressions of the same general tendency for autoimmunity in this mouse line.

SLE is a heterogeneous and unpredictable disease. In order to better understand the role of IFN-γ and its receptor in the pathogenic mechanisms underlying this disease, NOD mouse lines bearing targeted mutations for *Ifngγ* (NOD.*Ifng*$^{-/-}$) and the β chain of *Ifnγ* receptor (NOD.*Ifngr2*$^{-/-}$) were utilised.

6.2.2 Results

6.2.2.1 BCG mediates early precipitation of lupus in NOD mice.

NOD mice spontaneously develop organ-specific autoimmunity against insulin producing islet cells, leading to diabetes. They also display some features of systemic autoimmunity such as anti-nuclear autoantibodies and the development of haemolytic anaemia in senescence. *M.bovis* can trigger an early precipitation of systemic autoimmunity in NOD mice (Baxter et al., 1994; Hawke et al., 2003). It was interesting to examine whether immunisation with heat-killed, attenuated strain of *M.bovis* that prevented autoimmune diabetes development (Figure 6.1B), would induce any other form of autoimmunity in NOD mice. Female NOD mice of 8-weeks were i.v. injected with 0.25mg, 0.5mg and 1mg heat-killed BCG, and blood collected at monthly intervals to determine levels of haematocrit, haemoglobin and presence of Coombs' antibodies. Early precipitation of a systemic autoimmune disease similar to SLE was observed in 50% (4/8) of mice, which received 1mg BCG (Figure 6.15A). Lupus like autoimmune disease in NOD mice was characterised by Coombs' positive haemolytic anaemia (HA, Figure 6.15B), lowered haematocrit and haemoglobin levels in blood (Figure 6.15C and D), presence of anti-nuclear antibodies (ANA) in sera (Figure 6.15 E) and immune complex (IC) deposition in renal glomeruli (Figure 6.15 F). Mice that received lower doses of BCG (0.25mg or 0.5mg) failed to precipitate any of the above signs of autoimmunity (Figure 6.15A, C, D and Table 6.7).

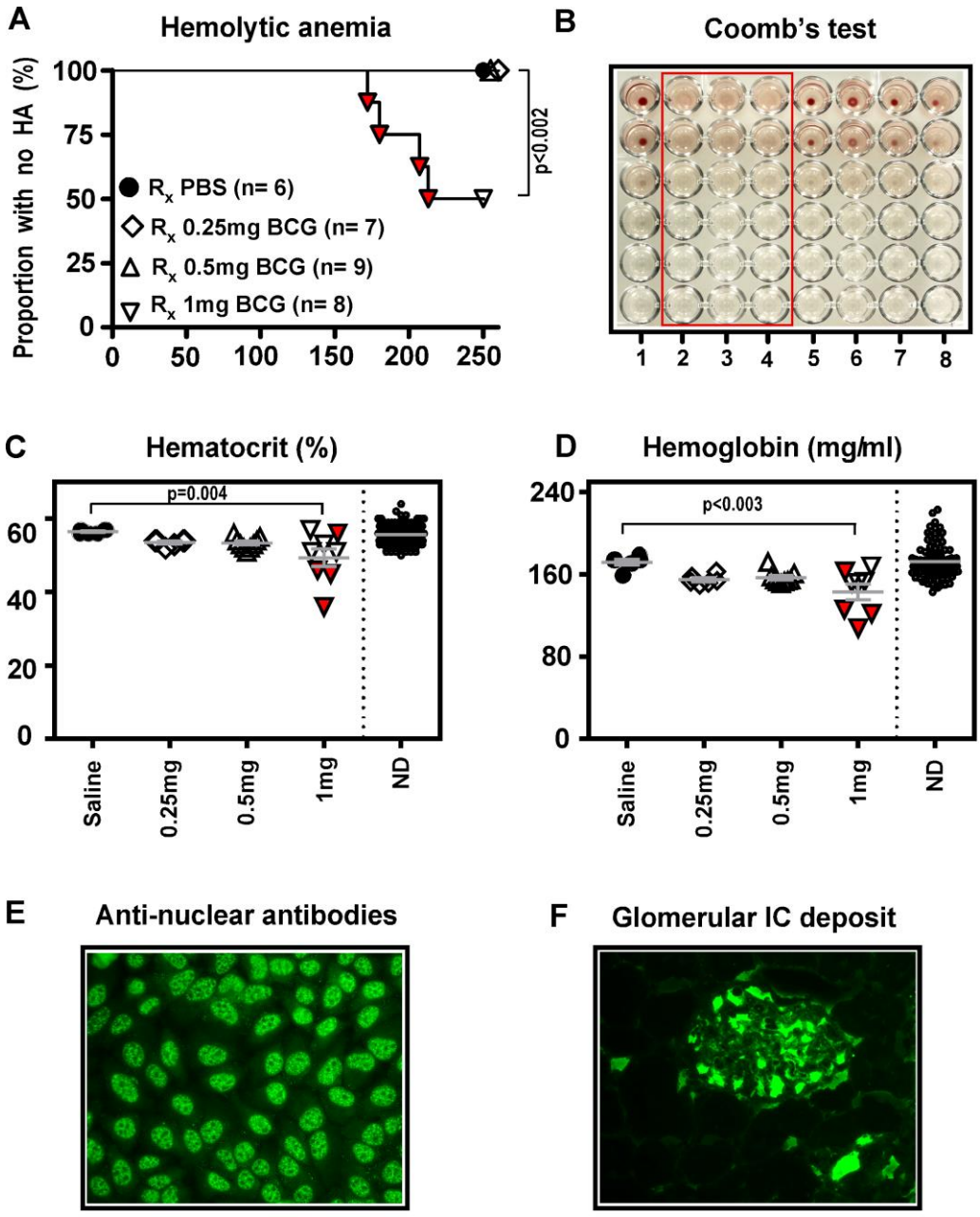


Figure 6.15 Survival of female NOD/Lt mice treated with various doses of BCG (A). Inverted red triangles represent mice that displayed characteristics of lupus. Direct Coombs' test for AIHA (B), columns 2, 3 and 4 on the microtiter plate are representative of Coombs' positive anti-RBC antibodies. Haematocrit (C) and haemoglobin (D) levels in NOD mice treated with various doses of BCG. Haematocrit and haemoglobin levels dropped significantly in response to an increase in BCG dose ($p<0.007$ and $p<0.005$ respectively, Kruskal-Wallis test). However, the most significant drop in haematocrit and haemoglobin levels were seen in mice that received *(contd.)*

1mg BCG when compared to saline controls (p=0.004 and p<0.003 respectively, Dunn's multiple comparisons test, multiplicity adjusted).Direct immunofluorescent detection of ANA in sera of BCG administered mice (E) and immune complex deposition in the renal glomerulus of mice that developed SLE like autoimmunity (F). ND: normal distribution in untreated controls (n=117).

NOD/Lt	Haematocrit (%)	Haemoglobin (mg/ml)
Saline (n=6)	56.5 ± 0.55	171.8 ± 6.8
0.25mg (n=7)	53.50 ± 0.84	155 ± 4.73
0.5mg (n=9)	53.33 ± 1.58	156.9 ± 6.39
1mg (n=8)	49.38 ± 6.87 *	143.0 ± 21.52 *
ND (n=117)	55.63 ± 2.71	172.5 ± 15.01

Table 6.7 Haematocrit and haemoglobin levels in NOD peripheral blood (Mean ± SD). * p=0.004 (haematocrit) and p<0.003 (haemoglobin), Dunn's multiple comparisons test). ND: normal distribution of values in untreated control mice.

6.2.2.2 Genetic ablation of *Ifng* or its signalling receptor prevented the onset of BCG-induced autoimmunity in NOD mice.

To determine whether IFN-γ or its downstream-signalling played a role in BCG mediated early precipitation of systemic autoimmunity in NOD mice, blood from saline treated NOD/Lt, NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice were compared with that from mice immunised with either 0.25mg or 1mg BCG. Mice were bled at monthly intervals for the detection of Coomb's antibodies and to assess haemolytic anaemia (by estimating haematocrit, haemoglobin levels). By 35-week age, 40% (4/10) NOD/Lt mice in the 1mg treatment group developed Coomb's antibodies,

while none of the IFN-γ or IFN-γR2 deficient mice tested positive for anti-red cell antibodies (Fig. 6.16 A, p<0.002, Mantel-Cox test). Haematocrit levels significantly dropped in all three groups of mice treated with 1mg BCG when compared to saline controls (p<0.02 (NOD/Lt), p<0.0002 (NOD.*Ifng*$^{-/-}$), p<0.008 (NOD.*Ifngr2*$^{-/-}$) Mann Whitney U test, Figure 6.16 B, Holm-Sidak's multiple comparisons test). However, while haemoglobin levels dropped significantly in 1mg BCG treated WT mice (p<0.003 Mann Whitney U test, Holm-Sidak's multiple comparisons test), the levels in NOD.*Ifn-γ*$^{-/-}$ and NOD.*Ifn-γr2*$^{-/-}$ mice remained unchanged (Figure 6.16 C). The average haematocrit and haemoglobin levels for all treatments in the three groups of mice at 30 weeks of age are listed in Table 6.8. The above findings illustrate that the genetic ablation of IFN-γ or its signalling receptor prevent the onset of BCG-induced lupus, suggesting an essential role for this cytokine in precipitating systemic autoimmunity in NOD mice.

	Haematocrit			**Haemoglobin**		
	Saline	**0.25mg**	**1mg**	**Saline**	**0.25mg**	**1mg**
NOD/Lt	56.50 ± 0.55	54.55 ± 1.76	49.38 ± 6.87	171.8 ± 6.8	158.3 ± 6.38	143 ± 21.52
NOD.*Ifng*$^{-/-}$	52.89 ± 2.26	55.33 ± 1.57	46.64 ± 3.34	156.5 ± 14.33	162.7 ± 13.61	151.2 ± 13.01*
NOD.*Ifngr2*$^{-/-}$	53.63 ± 2.07	52.10 ± 1.60	48.43 ± 4.73	163.2 ± 11.63	163.6 ± 18.57	167.3 ± 14.98*

Table 6.8 Peripheral blood haematocrit and haemoglobin levels (Mean ±SD). * p<0.05 compared with WT NOD mice (Mann Whitney U test).

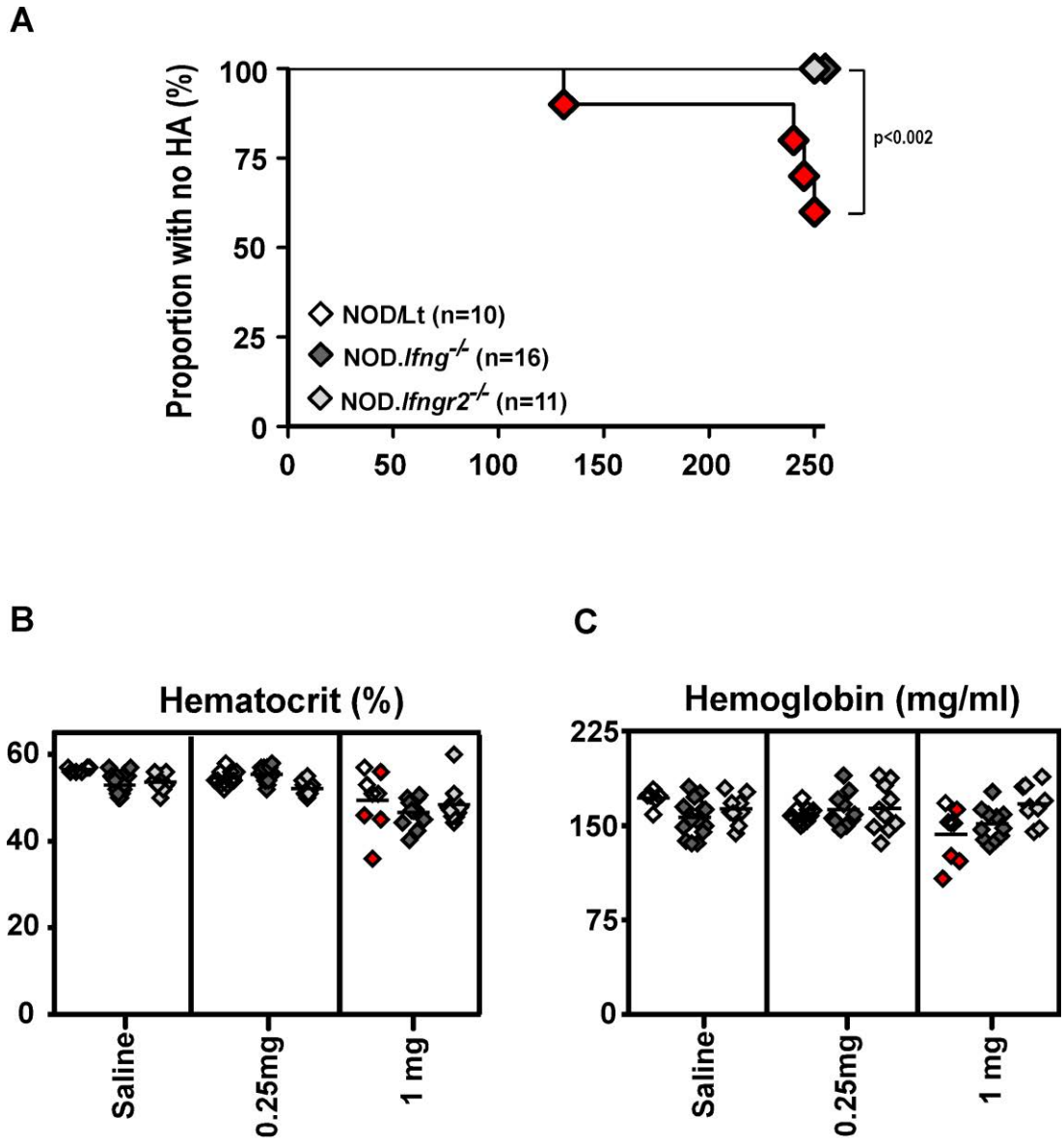


Figure 6.16 Survival of female NOD/Lt, NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice treated with 1mg BCG (A). Haematocrit (B) and haemoglobin (C) levels in BCG treated mice and saline controls. While 4 out of 10 NOD/Lt mice developed Coombs' antibodies (depicted as red diamonds), none of the IFN-γ and IFN-γR2 deficient mice did. Red diamonds in B and C represents mice that developed Coombs' antibodies.

6.3 Discussion

The incidence of spontaneous diabetes in female NOD mice housed under tightly controlled environmental conditions at the SPF facility of Comparative Genomics Centre during the period of this study was over 70%. Similar to earlier findings of Harada, Kishimoto and Makino (Harada et al., 1990) who demonstrated diabetes protection in NOD mice immunised with live BCG at a young age, a single injection of 0.25mg ImmuCyst (Bacillus Calmette-Guérin/Connaught), a freeze dried preparation of an attenuated strain of *M. bovis*, nearly totally prevented diabetes in our NOD colony. While diabetes was adoptively transferred into irradiated naïve recipients of more than $15x10^6$ diabetogenic splenocytes, BCG immunisation almost completely protected the recipients, suggestive of an inhibitory effect of mycobacteria on the efficacy of diabetogenic cells.

IFN-γ, a proinflammatory cytokine, has been acknowledged as a major contributor to T1D pathogenesis (Thomas et al., 2009). IFN-γ blockade by specific monoclonal antibodies (Campbell et al., 1991; Debray-Sachs et al., 1991) or soluble receptors (Nicoletti et al., 1996) in NOD mice, reduced spontaneous diabetes as well as diabetes induced by either cyclophosphamide or adoptive transfers. On the contrary, data from other studies indicate that autoimmune diabetes development in NOD mice does not require IFN-γ signalling (Hultgren et al., 1996; Kanagawa et al., 2000; Nicoletti et al., 1996; Serreze et al., 2000; Thomas et al., 1998). NOD mice

with functionally inactivated gene encoding IFN-γ, developed diabetes later and at a lower incidence (Hultgren et al., 1996; Serreze et al., 2001). However, in our environment, we found that susceptibility to spontaneous autoimmune diabetes in NOD.*Ifng*$^{-/-}$ mice was either equal (in females) or slightly higher (in males) than in parental NOD/Lt mice (Figure 6.4 A and B). It was likely that the naturally high penetrance of diabetes in female NOD mice could have potentially masked any evidence of an enhancement of this trait; however, a pro-diabetic effect of targeted deletion of IFN-γ was clearly seen after adoptive transfer (Figure 6.6 A). These findings raised the possibility that IFN-γ played a suppressive role in T1D development. This observation is in accordance with earlier studies that demonstrated diabetes inhibition in NOD mice being associated with IFN-γ-producing T cells, or administration of recombinant IFN-γ (Akhtar et al., 1995; Sobel et al., 2002; Zekzer et al., 1997). If the above hypothesis of an inhibitory role for IFN-γ in T1D development was true, it might be expected that any blockage of IFN-γ signalling would exacerbate the disease in NOD mice. However, in a previous study, Serreze and colleagues reported that despite a slight delay in diabetes onset in NOD.*Ifngr2*$^{-/-}$ mice, the cumulative incidence of long standing diabetes in these mice were similar to WT (Serreze et al., 2000). Intriguingly, we found (in two separate experiments, Figures 6.4 A and 6.5 A) that diabetes incidence in NOD mice with the functionally inactivated gene encoding the β-chain of the IFN-γ receptor was significantly reduced. This finding was in agreement with an earlier report of diabetes resistance in NOD mice carrying a null mutation at the IFN-

γ receptor α locus (Wang et al., 1997). However data from a subsequent study suggested that a closely linked diabetes resistance allele transferred to the NOD background from the 129 donor strain conferred the disease protection reported by Wang and colleagues, rather than the functionally inactivated IFN-γ receptor α chain (Kanagawa et al., 2000). As it was possible that the diabetes resistance observed in our NOD.*Ifngr2*$^{-/-}$ colony is affected by loci linked to the targeted gene, genetic homogeneity with the parental NOD/Lt strain was confirmed by typing 687 single nucleotide polymorphisms (Australian Genome Resource Facility, Melbourne, Australia) in random representatives. Of the 5 NOD.*Ifngr2*$^{-/-}$ mice that were typed, none differed from the parental strain. Having possibly excluded the risk of genetic contributions from donor strain conferring diabetes resistance in our NOD.*Ifngr2*$^{-/-}$ mice, there were two possible explanations for our incidence data in these mice. i) IFN-γ plays an important pro-diabetogenic role (this assumption is based on the reduced diabetes incidence observed in NOD mice with functionally inactivated gene encoding the β-chain of the IFN-γ receptor), or, paradoxically, ii) IFN-γ played an inhibitory role in NOD diabetes development. The second presumption arise from the possibility that IFN-γ could signal through alternate molecule(s), thereby conferring some level of resistance against autoimmune diabetes in mice that lack the β-chain of the IFN-γ receptor. However, an extensive literature search did not identify any alternate receptor for IFN-γ signalling, and any further study to identify such a factor lies beyond the scope of this thesis. In a recent study, Yi and colleagues employed an adoptive transfer

model in which β-cell specific $CD8^+$ but not $CD4^+$ effector T cells were successful in transferring diabetes into IFN-γR2 deficient NOD.*scid* mice (Yi et al., 2012). The lack of pathogenicity of $CD4^+$ effector T cells was attributed to the resistance of IFN-γR2 deficient β-cells to inflammatory-cytokine-induced cell-death. Thus, the inherent resistance of IFN-γR2 deficient β-cells to proinflammatory cytokine-induced destruction could be the most likely explanation for the partial diabetes protection observed in our NOD.*Ifngr2*$^{-/-}$ colony (Figure 6.17).

We have demonstrated that a low dose BCG immunisation (0.25mg) provided near total protection against diabetes in NOD mice, a finding in agreement with previous reports (Harada et al., 1990; Serreze et al., 2001). However, at the same dose, BCG did not protect targeted mutant NOD.*Ifn-γ*$^{-/-}$ mice and NOD.*Ifn-γr2*$^{-/-}$ mice from spontaneous diabetes or from disease induced in an adoptive transfer model, suggesting that IFN-γ signalling through its receptor is indispensable in BCG-mediated protection of NOD mice from autoimmune diabetes (Figures 6.5 A and B, 6.6 A and B). The temporal elevation of serum titres of IFN-γ in BCG treated NOD mice that were protected against T1D further raised the possibility of this cytokine being associated with mycobacteria-mediated immune protection.

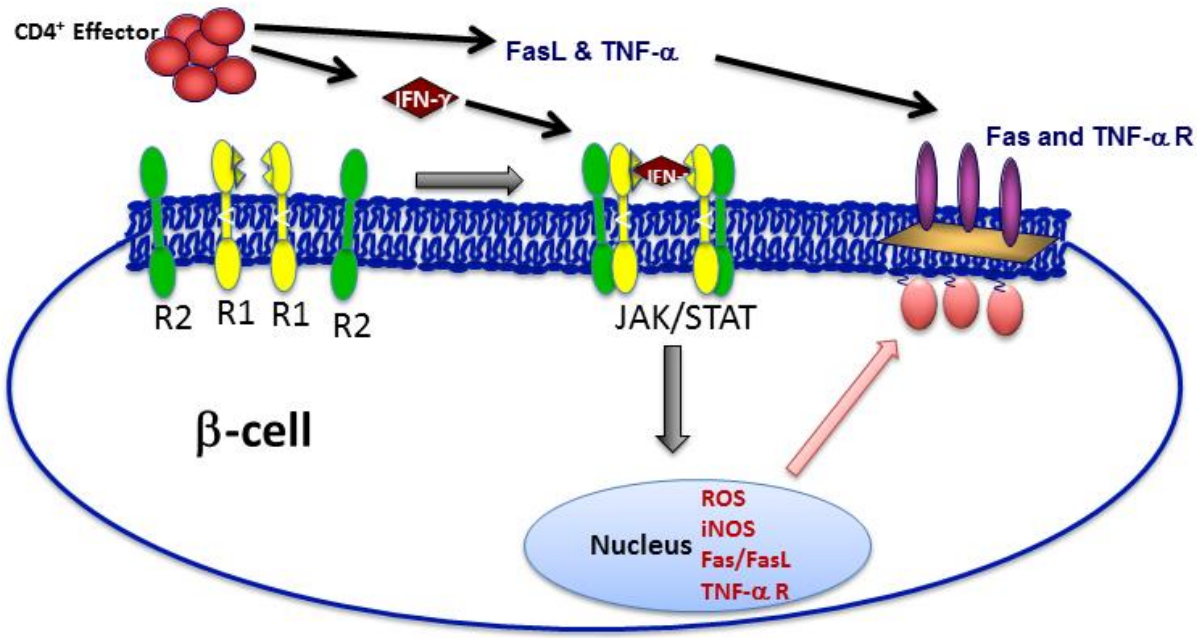


Figure 6.17 Possible mechanism of inhibition of spontaneous autoimmune diabetes in NOD.*Ifngr2*$^{-/-}$ mice. Inherent resistance of IFN-γR2 deficient islet b-cells to proinflammatory cytokine-induced destruction might play a role in providing the partial protection against diabetes seen in NOD.*Ifngr2*$^{-/-}$ mice (ref). However, around 50% of IFN-γR2 deficient NOD mice developed diabetes, which might be because lack of IFN-γR2 expression on β-cells prevents CD4^{+} effector T cell mediated damage, and not CD8^{+} cytotoxic T cell mediated death.

This conclusion is in agreement with earlier studies in which CFA or *M.bovis* treated NOD.*Ifng*$^{-/-}$ mice were poorly protected from autoimmune diabetes (Serreze et al., 2000), and anti- IFN-γ mAb administration eliminated diabetes protection offered by *M.bovis* in an adoptive transfer model of this disease. Qin and colleagues demonstrated that the inhibitory effect of mycobacteria on the efficacy of diabetes transfer could be partially replicated by the administration of IFN-γ (Qin et al., 2004). Inasmuch as exacerbation of diabetes in IFN-γ deficient NOD mice and the necessity of this cytokine in BCG-mediated protection of NOD mice from diabetes, it appears that this cytokine can play a role in suppressing autoimmune diabetes development.

The increased susceptibility of diabetes or the inability of BCG to mediated disease suppression in NOD mice carrying the mutant alleles of *Ifn-γ* and *Ifn-γr2* were not due to defects in the numbers of immunoregulatory cells, as genetic ablation of IFN-γ-or the β-chain of its receptor did not create a deficiency in proportions of CD4^{+}CD25^{+}FoxP3^{+} Tregs or αGal-Cer/CD1d-tetramer^{+} NKT cells in peripheral lymphoid organs of NOD mice (Figures 6.7 and 6.8 A). In fact, we observed significantly higher proportions of Tregs in the spleens of NOD.*Ifng*$^{-/-}$ mice and NOD.*Ifngr2*$^{-/-}$ mice and in the livers of NOD.*Ifng*$^{-/-}$ mice than in spleens and livers of parental NOD/Lt mice, suggesting a role for IFN-γ in Treg homeostasis. This finding is in agreement with previous studies that demonstrated an inhibitory role for IFN-γ in the induction of FoxP3^{+} Tregs (Chang et al., 2009; Wang et al., 2006;

Wei et al., 2007). However, NOD.*Ifng*$^{-/-}$ mice, which had more Tregs than WT NOD mice, were equally, or more (in males) susceptible to diabetes, indicating that the increased proportion of these regulatory cells did not have any inhibitory effect on diabetes development in mice that lacked IFN-γ signalling. Even as previous reports suggest the involvement of Tregs in mycobacteria mediated diabetes protection (Manirarora et al., 2008; Qin et al., 1993), data from a more recent study by Mori and colleagues indicated that CFA caused no change in the proportions or numbers of Tregs in peripheral lymph nodes of NOD mice with non-functional IFN-γ signalling (Mori et al., 2009). They further demonstrated that T cells lacking IFN-γ signalling were resistant to the suppressive effects of Tregs both *in vitro* and *in vivo*, suggesting that IFN-γ signalling is crucial for the suppression of pathogenic T cells via Tregs. Thus, despite a boost in proportions of Tregs observed in our mice that lacked IFN-γ, the inability of these cells to confer any inhibitory effect on diabetes development in NOD.*Ifng*$^{-/-}$ mice might be due to the above suggested resistance of IFN-γ deficient T cells against the suppressive effects of Tregs.

Even while the results discussed so far suggest a suppressive role for IFN-γ signalling on autoimmune diabetes as well as in BCG mediated diabetes protection in NOD mice, the precise mechanism(s) by which this cytokine confers disease protection remains unclear. 0.25mg BCG treatment offered no protection against diabetes in the IFN-γ and the IFN-γR2 deficient NOD mice, while it protected WT

NOD mice from developing the disease. A flow cytometric examination revealed significantly higher proportions and numbers of effector T cells ($CD44^{hi}CD62L^{lo}$) in the secondary lymphoid organs of BCG immunised NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice than in BCG treated WT NOD mice. Therefore, it appears that BCG-mediated IFN-γ production caused the suppression or elimination of activated T cells in NOD mice. This finding is supported by those of Qin and colleagues who demonstrated that BCG induced the production of TNF-α and IFN-γ, which selectively eliminated diabetogenic T cells via FAS or TNF pathways, in NOD mice (Qin et al., 2004). Kodama and colleagues in an *in vitro* islet cytotoxicity assay, found that CFA treatment selectively killed $CD45RB^{low}$ cells (activated or memory T cells) isolated from NOD mice, while it spared cells that were $CD45RB^{high}$ (naïve T cells) (Kodama et al., 2005), moreover T cells from NOD mice that lacked IFN-γ signalling were found to be resistant to Treg-mediated suppression in an adoptive transfer experiment (Mori et al., 2009). In a recent clinical trial, Faustman and colleagues reported BCG vaccination in T1D patients to cause a rapid increase in circulating insulin-autoreactive T cells that were mostly dead (Faustman et al., 2012). Taken together, the above findings suggest that one of the probable mechanisms by which mycobacterial components confers diabetes protection in NOD mice is by mediating the relatively selective elimination or suppression of islet autoreactive T cells, via mechanisms regulated by cytokines, including IFN-γ and TNF-α (Figure 6.18).

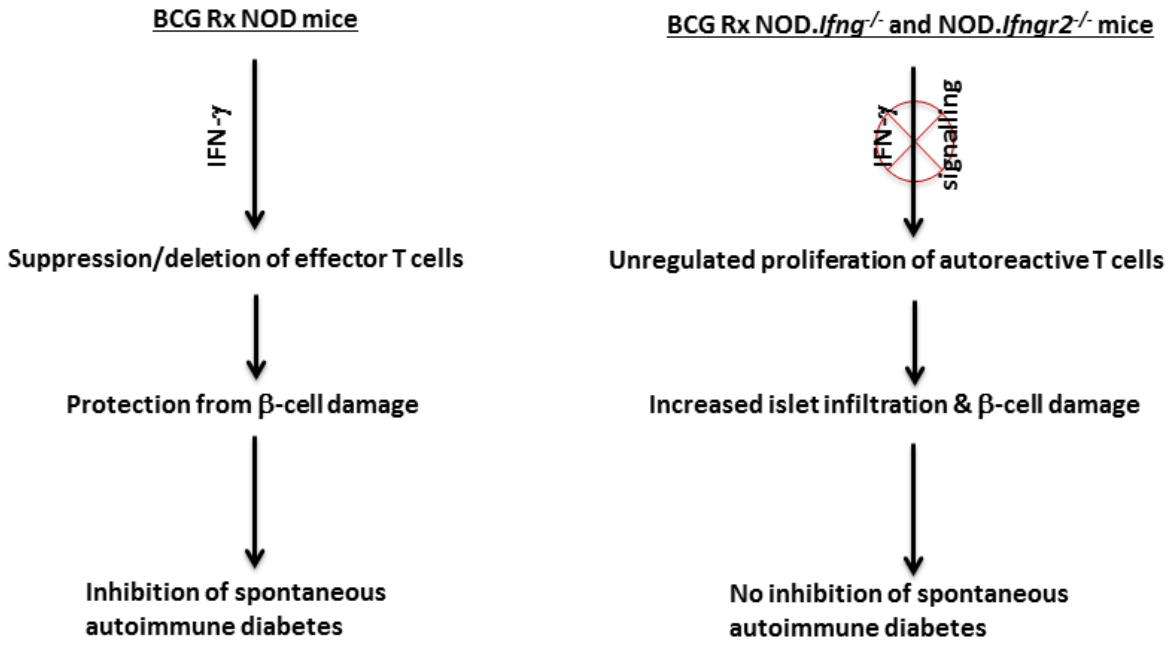


Figure 6.18 Simplified mechanism of BCG mediated inhibition of spontaneous diabetes in NOD mice. IFN-γ production is the key for BCG-mediated diabetes protection, and any deficiency of IFN-γ or its signalling receptor abrogates the protection offered by BCG against diabetes in NOD mice.

T cells deficient in IFN-γ signalling were shown to polarise towards an IL-17 producing pathogenic T cell population (Komiyama et al., 2006). Expansion of IL-17 producing T cells has been reported in pancreatic lymph nodes and peripheral blood of T1D patients (Ferraro et al., 2011; Honkanen et al., 2010). IL-17 was found to be highly expressed in the pancreas of autoimmune diabetes prone NOD mice (Vukkadapu et al., 2005). BDC 2.5 T cells that were polarised to an IL-17 producing T cell population induced autoimmune diabetes in NOD.*scid* mice (Martin-Orozco et al., 2009) and treatment with anti-IL-17 mAb prevented development of diabetes in NOD mice (Emamaullee et al., 2009). All these findings suggest an intimate association of this cytokine with T1D development. IFN-γ and IL-17 are known to counter-regulate each other (Cruz et al., 2006; Harrington et al., 2005; O'Connor et al., 2009) , and hence it might be expected that a deficiency in IFN-γ would enhance IL-17 levels. However, contrary to the expectation, we found no increase in proportions of IL-17 producing cells in the spleens of BCG treated NOD mice which lacked IFN-γ or the β-chain of its signalling receptor. Instead, BCG immunised NOD/Lt mice had significantly higher numbers of IL-17 expressing cells in their spleens than in NOD mice deficient in IFN-γ signalling. The higher proportions of IL-17 secreting cells observed in BCG treated NOD mice was in accordance with the findings of Scriba and colleagues, who demonstrated that stimulation of human $CD4^+$ T cells with mycobacterial products including BCG caused an elevation in levels of IL-17 (Scriba et al., 2008). Our observation suggests an association between IL-17 and IFN-γ in BCG mediated immune

response in NOD mice. Genetic ablation of *Il-17a* or silencing of IL-17 by RNA interference in NOD mice had no effect on the frequency of spontaneous or cyclophosphamide-induced diabetes (Joseph et al., 2012; Kuriya et al., 2013), suggesting that IL-17 is not essential in the pathogenesis of autoimmune diabetes. Diabetogenic potential of NOD splenocytes remained unaltered even after polarisation to an IL-17 producing phenotype (Nikoopour et al., 2008). Th17 polarised splenocytes from CFA treated NOD mice delayed diabetes development in NOD.*scid* recipients of NOD diabetogenic splenocytes, and this protection was reduced when T cells treated with anti-IL-17 mAb were used for transfer (Nikoopour et al., 2010). These findings suggested a role for IL-17 in mycobacteria mediated diabetes inhibition in NOD mice. Data from a recent study demonstrated a collective role for IL-17A and IFN-γ in inhibiting chlamydial lung infection in mice (Zhang et al., 2012). In light of the above mentioned association of IL-17 with IFN-γ, and their suppressive roles in NOD autoimmune diabetes, it is possible that IL-17 acts as an accessory for IFN-γ-mediated diabetes protection conferred by BCG. This hypothesis is in agreement with a recent finding that reported a concomitant rise in the levels of IFN-γ and IL-17, with protection against diabetes induced by mycobacteria in NOD mice (Nikoopour et al., 2010). Thus, it appears that adjuvant mediated diabetes protection in NOD mice relies on a synergistic action of IFN-γ and IL-17 cytokines.

NK cells are crucial in the initial response against mycobacterial infections (de la Barrera et al., 2004; Wolfe et al., 1976), and these cells are the predominant producers of IFN-γ, a crucial cytokine in the innate immune response (Schoenborn and Wilson, 2007; Yang et al., 1995). NK cells are believed to be one of the earliest infiltrators of the pancreatic islets in NOD mice and these cells have also been detected in the pancreas of diabetic patients (Brauner et al., 2010; Dotta et al., 2007; Feuerer et al., 2009; Poirot et al., 2004). IFN-γ plays a crucial role in promoting NK cell activity (Boehm et al., 1997), and IFN-γ secreted by activated NK cells has been reported to activate auto-reactive T cells, unleashing them to aggressively attack and destroy pancreatic islets in NOD mice (Feuerer et al., 2009). Thus, it appears that NK cell activation and subsequent release of IFN-γ cytokine by these cells is crucial in the precipitation of autoimmune diabetes.
In agreement with a pro-diabetogenic role for NK cells, we found that depletion of NK cells from splenocytes of diabetic NOD mice, significantly delayed the transfer of disease into NOD.*scid* recipients (Figure 3.9B). Contrary to a pro-diabetogenic role for these cells, impaired NK cell function was observed in the blood of diabetic patients and in lymphoid tissues of NOD mice, which spontaneously develop T1D (Carnaud et al., 2001; Rodacki et al., 2007). Recently, Beilke and colleagues demonstrated that though antibody mediated NK cell depletion in NOD mice caused a slight delay in spontaneous and adoptively transferred diabetes, the cumulative incidence of long standing diabetes in these mice was similar to WT NOD mice (Beilke et al., 2012). These findings raise some doubts over the role for

NK cells in T1D onset and progression. To add to this confusion, several studies suggest a protective role for NK cells in T1D. Lee and colleagues demonstrated that mycobacterial components increased the frequency and cytotoxicity of NK cells in blood and spleen, and that these cells are needed for CFA mediated diabetes protection in NOD mice (Lee et al., 2004). The same group later demonstrated that NK cells protected CFA treated NOD mice against diabetes via the secretion of IFN-γ (Lee et al., 2008). Contrary to the findings of Lee and colleagues, we observed that BCG treated NOD.*scid* mice were protected from diabetes irrespective of whether they received normal or NK-depleted splenocyte preparations, indicating a limited role for NK cells in BCG mediated diabetes protection. Thus, the role of NK cells in the induction of spontaneous diabetes or its role in mycobacteria mediated diabetes protection, still remains controversial. We found that NK cell depletion significantly delayed the transfer of disease into NOD.*scid* recipients, but the fact that over 85% of recipients in the NK depleted model developed the disease indicates interplay of additional factor(s) in NOD diabetes development. The role for NK cells in BCG mediated diabetic protection appeared to be minimal, as the depletion of this innate immune cell-type did not alter the protective effect of BCG in an adoptive transfer model of the disease. However, our findings had its limitations. The resident NK cells in NOD.*scid* recipients, albeit reduced in numbers and functions, could have abrogated the effects of NK cell depletion from donor cell preparation to some extent.

Toll like receptors are pattern recognition receptors expressed by cells in the immune system and play a major role in identifying microbes and microbial constituents. Previous studies have shown that TLR2, which recognizes lipopolysaccharides (Lien et al., 1999), a major constituent of mycobacterial cell wall, can both exacerbate and prevent initiation of T1D (Karumuthil-Melethil et al., 2008; Kim et al., 2007). A polymorphism in the TLR2 gene was recently found to be significantly associated with autoimmune diabetes development in children (Bjornvold et al., 2009). These findings suggest that TLRs can modulate autoimmune β-cell destruction. We observed that spontaneous diabetes incidence in NOD.*Tlr2*$^{-/-}$ mice was significantly lower than that in WT littermates. Our finding was in accordance with the results of Kim and colleagues, who reported that diabetes incidence in NOD mice was reduced by about half in TLR2 deficient mutants (Kim et al., 2007). These findings suggest that signalling through this receptor was crucial in diabetes initiation. However, there exists the possibility that an independent diabetes resistance gene conferred diabetes protection in TLR2 deficient NOD mice, as the *Tlr2* locus maps to the same chromosome 3 region as the *Idd17* diabetes susceptibility locus (Podolin et al., 1997). Purified mycobacterial cell-wall components have been shown to preferentially activate TLR2; however we found that NOD.*Tlr2*$^{-/-}$ mice injected with BCG were equally protected from autoimmune diabetes as WT NOD mice, suggesting that mycobacterial components might be signalling through alternative TLRs or that their activity includes mechanisms independent of TLR signalling. Consistent with

this hypothesis Wong and colleagues found that ablation of TLR9 markedly reduced autoimmune diabetes in NOD mice (Wong et al., 2008). Data from an earlier study reported TLR4 to regulate host immune response against BCG in ways distinct to that of TLR2 (Heldwein et al., 2003). Lee and colleagues recently demonstrated that immune cells were activated by CFA in NOD.*MyD88*$^{-/-}$ mice, suggesting that mycobacterial components could induce immune response independent of TLR signalling as well (Lee et al., 2011).

Although 0.25mg BCG treatment offered no protection against diabetes in the IFN-γ and the IFN-γR2 deficient NOD mice, the higher dose of 1mg, prevented diabetes in both IFN-γ-deficient NOD mice and in mice deficient in β-chain of its receptor, suggesting that, at a higher dose, IFN-γ-independent mechanism(s) might come into play. BCG exposure has been reported to precipitate systemic autoimmune responses typical of SLE in NOD mice (Baxter et al., 1994). In accordance with the above report, we found that NOD mice administered 1mg BCG developed lupus like autoimmune disease, characterised by Coombs' positive haemolytic anaemia, lowered haematocrit and haemoglobin levels in peripheral blood, raised titres of ANA in sera and IC deposition in renal glomeruli, consistent with glomerulonephritis. However, the deficiency of IFN-γ or its receptor prevented the disease precipitation in NOD mice. This is in agreement with an earlier study which demonstrated that deletion of IFN-γ receptor significantly reduced disease development in lupus prone (NZB x NZW) F1 mice (Haas et al., 1998). The above

findings suggest that even as BCG-induced production of IFN-γ played a crucial role in diabetes protection in autoimmune prone NOD mice, the excessive production of this cytokine might have switched the expression of a predisposing tendency to other forms of autoimmunity in NOD mice.

In Summary, findings in this chapter indicate that environmental agent can have either a potentiating or an inhibitory role in the development of autoimmunity. M.*bovis*-induced IFN-γ-secretion abrogated autoimmune diabetes in NOD mice; the same protein, however, played a role in precipitating another form of autoimmunity, indicating the pleiotropic nature of this cytokine. The timing and the location of expression of IFN-γ appeared to affect immune response in NOD mice.

……

CHAPTER 7

……

CONCLUDING REMARKS

7.1 Introduction

Studies on the pathogenesis of autoimmune diabetes are largely focused on the analysis of diabetogenic T cells, and most treatment strategies trialled to date attempted to ameliorate the effects of islet-specific T cells (Michels and von Herrath, 2011). However, majority of these strategies have had with limited success in preserving residual β-cell function, especially in humans (Staeva et al., 2013), due to factors including, the need for intervention at a very early stage of autoimmunity, and the need to target multiple specificities owing to epitope spreading (Shoda et al., 2005; Tian et al., 1997). An intrinsic dialogue between β-cells and the innate and adaptive immune cells causes pancreatic β-cell-stress. Distressed β-cells express FAS, secrete interferons as well as chemokines, including CXCL10/IP-10, that are capable of recruiting and activating autoreactive immune cells, indicating a role for β-cells in in the initiation and progression of autoimmunity. Better understanding on how β-cells influence the local immune response, will help in designing novel and effective therapeutic approaches for T1D.

In this study, I attempted to elucidate some of underlying mechanisms of NOD diabetes, with a particular focus on components of the innate immune system, which affected β-cell survival. The TRAIL-deficient NOD mouse strain (NOD.*Tnfsf10*$^{-/-}$) and the NOD lines exhibiting allelic FASL expression (NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2}), provided *in vivo* support for crucial

roles of TRAIL and FASL in controlling islet susceptibility to immune destruction in autoimmune diabetes. Results described in chapters 4 and 5 suggested that these members of the TNF-ligand-superfamily confer protection against diabetes by regulating islet secretion of chemoattractants and suppressor proteins associated with the disease. Results from experiments performed on targeted gene deficient mutants of *Ifng* and its β receptor, illustrated an important role for IFN-γ, a proinflammatory cytokine, in regulating the autoimmune destruction of pancreatic islets. Findings detailed in chapter 5 indicated that temporal and spatial modulation of this cytokine could limit β-cell damage, thereby inhibiting the development and pathogenesis of autoimmune diabetes.

7.2 TRAIL and FASL execute parallel functions in T1D

This study illustrated that two apoptosis-inducing-ligands belonging to the TNF ligand superfamily, TRAIL and FASL, can confer a protective effect against autoimmune diabetes in NOD mice. TRAIL-deficient NOD mice displayed accelerated diabetes and severe insulitis compared to WT NOD mice. A protective role for TRAIL in NOD diabetes has been acknowledged in recent literature. Protein mediated blockade of TRAIL exacerbated diabetes and insulitis in NOD mice (Lamhamedi-Cherradi et al., 2003a), conversely, adenoviral delivery of TRAIL in NOD mice significantly lowered its disease incidence (Kang et al., 2010). However, ours was the first demonstration of an increased incidence of spontaneous diabetes in NOD mice bearing a targeted deletion of TRAIL. Incidental

discovery of NOD mouse lines with allelic FASL expression revealed an unexpected and interesting role for this death ligand in autoimmune diabetes development. The NOD.*D1Bax208*^{m2} mouse line, carrying the low-FASL-expressing allele, displayed high diabetes incidence and severe insulitis compared to the lower disease incidence and modest islet infiltration observed in the NOD.*D1Bax208*^{m1} mouse line, which had the high-FASL-expressing allele. There is other evidence of a negative association between FASL-expression and diabetes susceptibility in NOD mice. Kayagaki and colleagues reported lower FASL expression in NOD mice, compared to nondiabetes-prone BALB/c mice (Kayagaki et al., 1997), and FASL overexpressing cells were instrumental in lowering the incidence and delaying the progression of NOD diabetes in adoptive transfer models (Franke et al., 2007; Kim et al., 2000) . Our data demonstrating an increased incidence of spontaneous diabetes in NOD mice carrying the low-FASL-expressing allele is consistent with the above findings, and provides more direct evidence for a protective role of this death ligand in autoimmune diabetes.

TRAIL and FASL are major contributors in intrathymic negative selection process and peripheral T cell regulation (Corazza et al., 2004; Griffith et al., 2007; Lamhamedi-Cherradi et al., 2003b; Watanabe-Fukunaga et al., 1992). Such a role for these death ligands could potentially account for the increased severity of spontaneous diabetes observed in NOD.*Tnfsf10*$^{-/-}$ and NOD.*D1Bax208*^{m2} mice. However, T cell proportions in the thymi and peripheral organs of these mice

appeared normal, eliminating the possibility of a lymphoaccumulation-mediated exacerbation of diabetes. Moreover, NOD.*scid* mice receiving cells from diabetic NOD.*Tnfsf10*$^{-/-}$ and NOD.*D1Bax208*^{m2} mice developed the disease at a rate similar to that of NOD.*scid*-recipients of diabetogenic cells from control mice, suggesting the lack of a systemic increase in numbers or activation of autoreactive T cells in these mutant mouse lines. The reciprocal transfer of diabetogenic splenocytes between NOD.*Tnfsf10*$^{-/-}$ and WT NOD mice indicated that accelerated diabetes was associated with TRAIL-deficient recipients, and not the genotype of the donors. Similar experiments with NOD.*D1Bax208*^{m1} and NOD.*D1Bax208*^{m2} mice revealed that accelerated diabetes was associated with low-FASL-expressing recipients, and not the genotype of the donors. These findings raised the possibility that expression of these TNF superfamily ligands in the nonhematopoietic compartment conferred protection against autoimmune diabetes in WT NOD mice.

Chemokines are important mediators of cell trafficking implicated in autoimmune diabetes development both in man (Eizirik et al., 2009) and in NOD mice (Rhode et al., 2005). It is possible that in the initial stages of T1D development, microbial products or constituents of β-cell apoptosis trigger, via islet-TLRs, the secretion of proinflammatory chemokines, especially IP-10, that are able to recruit and localise autoaggressive immune cells. These activated autoreactive cells also secrete high levels of IP-10 (Frigerio et al., 2002; Roep et al., 2010; Turley et al., 2003; Uno et al., 2010), thereby imprinting a pattern that perpetuates the autoimmune cascade.

This chemokine is also capable of directly triggering β-cell destruction (Schulthess et al., 2009), contributing to the pathophysiology of T1D. We found that the triggering of islet TLRs by LPS induced the production of IP-10 *in vitro*; however, TRAIL ligation suppressed the secretion of this proinflammatory chemotactic cytokine. Expression profiling of islet transcripts identified the downregulation of *Socs1* (encoding suppression of cytokine signalling (SOCS1)) in low-FASL-expressing NOD.*D1Bax208*^{m2} mice, which had accelerated diabetes and severe insulitis. SOCS1 is a negative regulator of proinflammatory effects of IFN-γ, and it prevents IFN-γ-mediated β-cell death (Alexander, 2002; Cottet et al., 2001; Flodstrom-Tullberg et al., 2003). SOCS1 also attenuates the pathogenic effects of the highly potent chemokine IP-10 (Barral et al., 2006). It was therefore possible that higher SOCS1 levels in the islets of NOD.*D1Bax208*^{m1} mice negatively regulated IFN-γ and IP-10-mediated β-cell destruction, in contrast to the severe destruction seen in the pancreatic islets of low SOCS1 expressing NOD.*D1Bax208*^{m2} mice. Similar to FASL, TRAIL signalling also increased SOCS1 expression in NOD pancreatic islets (Zauli et al., 2010), and upregulation of this cytokine-signalling-suppressor-protein was associated with the amelioration of autoimmune diabetes in these mice. Thus, the systematic characterisation of the mechanism of protection mediated by TRAIL and FASL in NOD mice revealed a remarkable parallel between the β-cell protective effects of these two homologous ligands (Table 7.1, Figure 7.1).

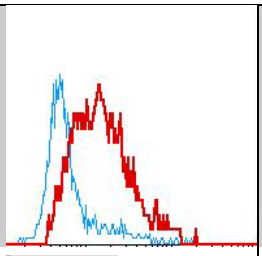
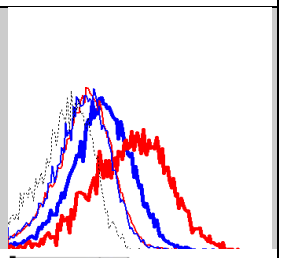
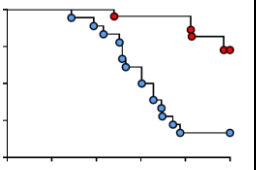
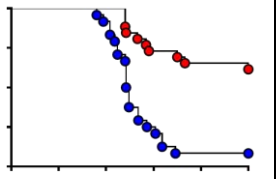
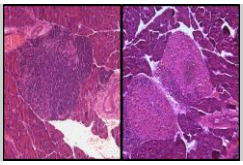
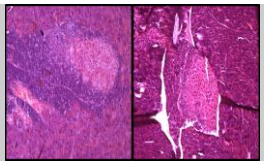
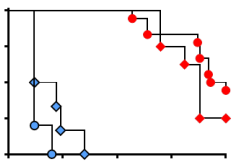
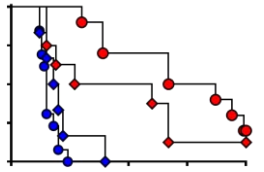
	NOD.*Tnfsf10*$^{-/-}$		NOD.*D1Bax208*^{m2}	
Expression levels	No TRAIL expression, (blue); constitutive expression in WT NOD/Lt (red)		Lower FASL expression in NOD.*D1Bax208*^{m2} (blue) compared to NOD.*D1Bax208*^{m1} (red)	
Diabetes incidence	Higher incidence in TRAIL KO (blue) mice than in WT.		Higher incidence in low FASL expressing NOD.*D1Bax208*^{m2} mice (blue)	
Severity of insulitis	Higher in TRAIL deficient mice (left) than in WT.		Higher in reduced FASL-expressing NOD.*D1Bax208*^{m2} mice (left)	
Reciprocal transfer	Higher rate of diabetes transfer into TRAIL deficient recipients (blue)		Higher rate of diabetes transfer into low FASL-expressing recipients (blue)	
Islet expression	No TRAIL expression	Higher islet secretion of chemokine IP-10 that promotes homing of islet infiltrating cells	Low FASL expression	Low islet expression of SOCS1, a strong inhibitor of cytokine (IFN-γ) signalling.

Table 7. 1 Remarkable similarities between the functions of TRAIL and FASL in NOD diabetes. In summary, TRAIL deficient NOD mice had severe islet infiltration, a finding comparable to that in low FASL-expressing NOD.*D1Bax208*^{m2} mice. It appears that a TRAIL deficient environment enhanced islet-derived-IP-10-mediated lymphocyte homing, while low FASL levels in islets supressed SOCS1, a strong inhibitor of IFN-γ and IFN-γ-induced proinflammatory chemoattractant IP-10, indicating that these two closely related ligands of TNF superfamily conferred protection to NOD islets through similar mechanisms.

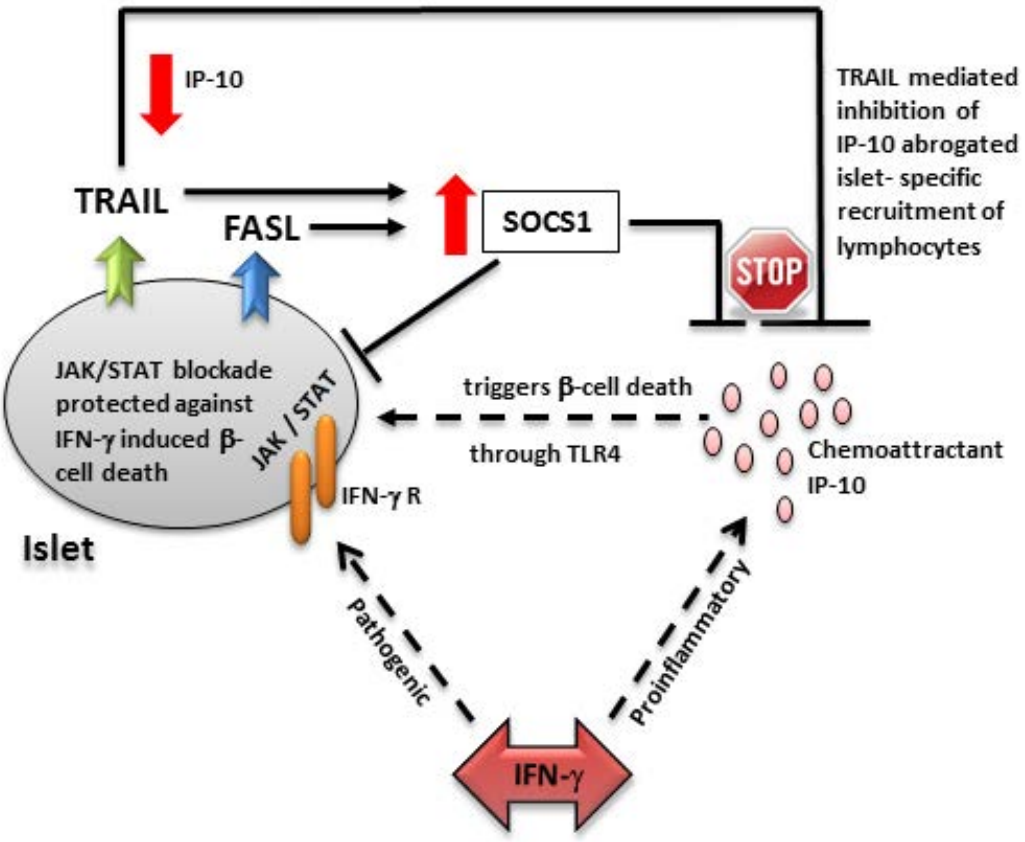


Figure 7.1 Cytokine/chemokine modulation by TRAIL and FASL, ligands of the TNF superfamily confers protection to islet cells. TRAIL inhibits islet secretion of IP-10, a potent chemoattractant of immune cells capable of secreting proinflammatory cytokines cells. IP-10 is also capable of directly triggering β-cell destruction. TRAIL/FASL expression by islets upregulate SOCS1, which acts as a feedback inhibitor regulating response to cytokines including IFN-γ, by interfering with the JAK/STAT signal transduction pathway. STAT-1-regulated genes are mainly pro-apoptotic in β-cells. SOCS1 also inhibits the expression of proinflammatory chemokines including IP-10. In addition to modulating the local cytokine/chemokine levels, TRAIL and FASL expression on islets provide immune privilege by binding to their respective death receptors on infiltrating cells, inducing their apoptosis.

7.3 IFN-γ is vital for BCG-induced diabetes protection in NOD mice

IFN-γ is an important mediator of β-cell death (Campbell et al., 1991; Debray-Sachs et al., 1991), and is acknowledged as a major contributor of T1D pathogenesis (Thomas et al., 2009). Transgenic mice overexpressing IFN-γ had severe β-cell destruction (Sarvetnick et al., 1988). In contrast, NOD mice genetically deficient in IFN-γ or its β-chain receptor remained susceptible to diabetes, suggesting that IFN-γ signalling is not essential in NOD islet autoimmunity (Hultgren et al., 1996; Serreze et al., 2000). In the work described here, we confirmed that genetic ablation of *Ifng* did not alter the incidence of spontaneous diabetes in NOD mice. Taken together, the contradicting observations on the role of IFN-γ in autoimmune diabetes suggest that, though this cytokine is involved in the progression from insulitis to β-cell death, in its absence, other mechanisms of islet cell destruction are sufficient. Interestingly, we found that NOD mice with functionally inactivated gene encoding the β-chain of *Ifng* receptor (NOD.*Ifngr2*$^{-/-}$) had significantly lower diabetes incidence compared to NOD.*Ifng*$^{-/-}$ and NOD/Lt mice. Experiments by Yi and colleagues indicated that β-cells deficient in IFN-γ β-chain-receptor had an inherent resistance to CD4 T cell-induced proinflammatory cytokine-mediated destruction (Yi et al., 2012). This could be one of the reasons behind the reduced incidence observed in NOD.*Ifngr2*$^{-/-}$ mice, another being a so far unknown ligand that signal though the β-chain receptor of IFN-γ, causing beta cell destruction.

Harada and colleagues reported a protective role for BCG in NOD diabetes (Harada et al., 1990). Corroborating the above finding, we observed that 0.25mg BCG injected i.v. into 8-week-old NOD mice provided near total protection against autoimmune diabetes. Whereas IFN-γ was not the only effector agent involved in the initiation and progression of autoimmune diabetes, it was definitely the key factor mediating BCG-associated disease suppression in NOD mice. Anti- IFN-γ monoclonal antibody treatment eliminated diabetes protection offered by BCG in an adoptive transfer model of this disease (Qin et al., 2004), consistent with this hypothesis. Furthermore, our findings indicate that deficiency in IFN-γ signalling eliminated the ability of BCG to protect NOD mice against spontaneous diabetes, as well as against adoptively transferred disease, were also consistent with a crucial role for this cytokine in BCG-mediated diabetes protection. BCG treated NOD.*Ifng*$^{-/-}$ and NOD.*Ifngr2*$^{-/-}$ mice had higher effector: naïve T cell ratio in the peripheral lymphoid organs, compared to BCG immunised parental NOD/Lt mice (Figures 6.9 and 6.10), indicating that BCG-induced IFN-γ-associated mechanisms are involved in the suppression or elimination of activated T cells. Our observation is in accordance with data that suggested selective elimination of diabetogenic effector cells by BCG related mechanisms, that played a role in the amelioration of autoimmune processes underlying T1D development (Faustman et al., 2012; Kodama et al., 2003; Qin et al., 2004; Ryu et al., 2001). Adoptive transfer experiments by Mori and colleagues revealed that T cells from IFN-γ deficient NOD

mice were resistant to Treg mediated suppression, consistent with a role for this cytokine in limiting effector T cell activity.

IFN-γ therefore exhibits paradoxical effects in T1D. IFN-γ signalling through STAT-1 regulated a number of genes that increased the sensitivity of β-cells to apoptotic stimuli, and mediated the secretion of proinflammatory chemokines including IP-10 (Moore et al., 2011). On the other hand, IFN-γ emerged as a key mediator in BCG-induced diabetes-inhibition in NOD mice. Timing and site of exposure of this cytokine might explain the variable effects observed. Among the initial events in T1D development, microbial products or molecular patterns released from apoptotic β-cells could ligate islet-TLRs (at around 2 week in NOD mice), resulting in the secretion of proinflammatory cytokines and chemokines, including IFN-γ and IP-10 (Giarratana et al., 2004; Kim et al., 2007; Turley et al., 2003). This proinflammatory chemotactic milieu would attract autoaggressive immune cells, especially IFN-γ-secreting, $CXCR3^+$ effector T cells (Loetscher et al., 1996; Sallusto et al., 1998), and induce β-cells to release more chemokines. This positive feedback loop could further attract additional mononuclear cells, perpetuating islet infiltration and subsequent autoimmune destruction of β-cells, in a partially IFN-γ-dependent manner (Figure 7.2 A). Results discussed in chapter 6 indicate that BCG instillation in prediabetic NOD mice (on or before 8 week of age) protected them from T1D, and that IFN-γ signalling was the unique critical factor in this model. This scenario of IFN-γ being proinflammatory in the initial stages of

islet infiltration, but conferring protection when induced by BCG at a later stage of insulitis, is reminiscent of the RIP-GP-TNF-α mouse model, in which early expression of the proinflammatory cytokine TNF-α enhanced the frequency of diabetes in mice, while a late expression reduced the disease (Christen et al., 2001). Similarly in the RIP-LCMV-NP mouse model although accelerated diabetes onset occurred 2-3 month post LCMV infection; a secondary infection 4 weeks later with LCMV-Pasteur, prevented T1D in this mouse model (Christen et al., 2004; Oldstone, 1988). High titres of LCMV-Pasteur were found in the pancreatic draining lymph nodes (PDLN) of these mice, and were associated with high expression of IP-10, reduction in islet infiltration and increased apoptosis of autoaggressive T cells in the PDLN. These findings suggested that the IP-10 chemokine-gradient caused islet effector T cells to traffic out of the pancreas and towards PDLN, the site of secondary inflammation. BCG is also capable of inducing the secretion of large amounts of IP-10 either directly, or indirectly through mechanisms controlled by BCG derived IFN-γ (Luo et al., 2007). These parallels between the LCMV and BCG-mediated inhibition of diabetes in NOD mice suggest deposits of live attenuated form of a heat-killed *M.bovis* (BCG) might have created a 'sink effect' that removed autoreactive T cells from the circulation and reversed the cellular traffic to the pancreatic islets (Figure 7.2 B). In accordance with the proposed peripheral 'sink effect' induced by *M.bovis* injection, BCG instillation ameliorated experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS), by diverting the traffic of myelin oligodendroglial

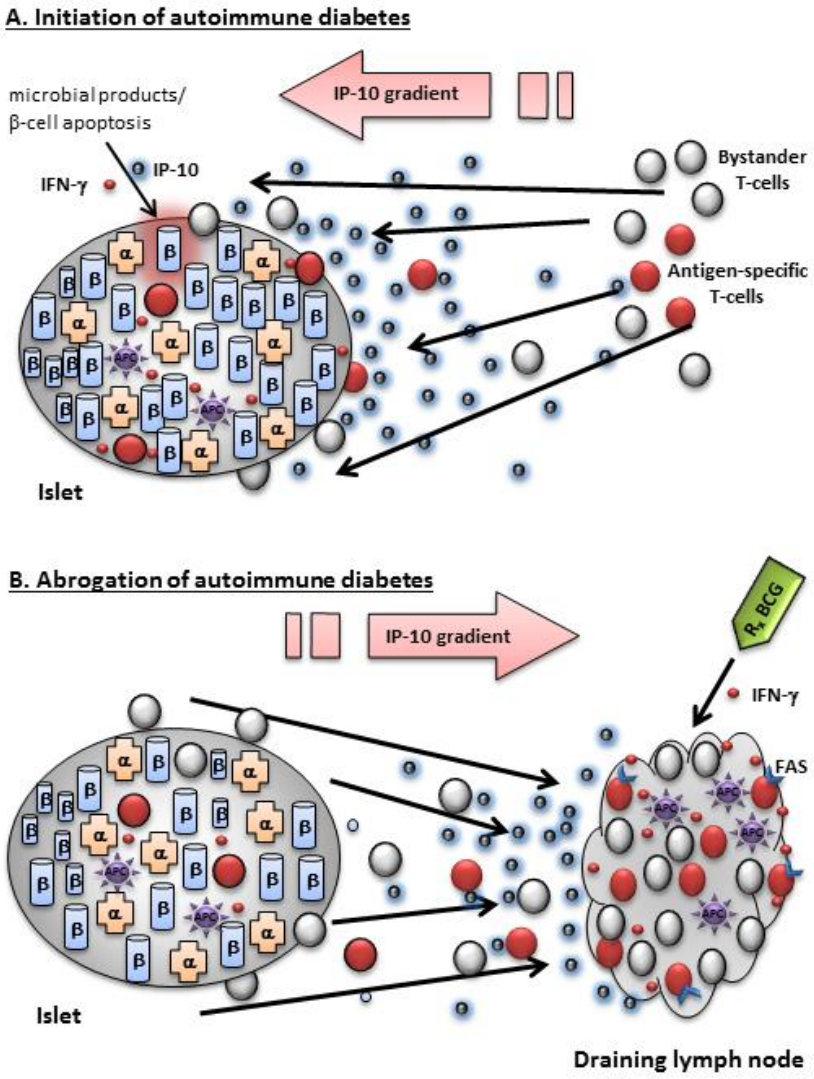


Figure 7.2 Proposed mechanism of BCG mediated inhibition diabetes in NOD mice. Triggering of islet TLRs by microbial products or components of dying β-cells upregulate secretion of proinflammatory chemokine IP-10 that attract activated T cells, macrophages, NK cells, DCs etc. These islet-infiltrating cells produce proinflammatory cytokines including IFN-γ and TNF, which induce β-cells to release more IP-10, creating a chemokine gradient that will attract additional mononuclear cells exacerbating β-cell destruction (A). Intravenous BCG administration at an early stage of insulitis interrupts this vicious cycle. It causes rapid accumulation of IFN-γ in the secondary lymphoid organs, thereby diverting the inflammation away from the pancreatic islets. IFN-γ-induced secretion of chemokines reverses the dynamics of immune cell migration, resulting in abrogation of NOD T1D. Additionally, IFN-γ selectively eliminates diabetogenic T cells via FAS or TNF pathways (B).

glycoproteinautoantigen (MOG)-specific T cells away from the central nervous system (Sewell et al., 2003). We also observed higher naïve: effector T cell ratios in the peripheral organs of BCG treated NOD mice compared to IFN-γ and IFN-γR2 deficient NOD mice. It was therefore possible that BCG mediated the elimination of effector T cells in an IFN-γ-dependent manner. Taken together, exposure of IFN-γ and IP-10 at the right location and at the right time could prove beneficial in inhibiting the ongoing autoimmune process, and might have relevance in the clinical context.

Though BCG conferred protection against autoimmune diabetes in NOD mice, at a high dose (1mg) it precipitated another form of autoimmunity with characteristics typical of SLE in some patients, including haemolytic anaemia, antinuclear antibodies in sera and immune complex glomerulonephritis (Figure 6.15). IFN-γ is vital for the development of autoimmunity in lupus prone MRL/I mice (Prud'homme et al., 1995). In accordance with the above finding, NOD mice deficient in IFN-γ signalling were spared from the BCG-mediated precipitation of systemic autoimmunity, suggesting the crucial role for IFN-γ in lupus development. SLE patients had high serum and plasma levels of the proinflammatory chemokine, IP-10, which strongly correlated with disease activity, moreover, infiltrating $CXCR3^+$ cells participated in a variety of manifestations of SLE (Fragoso-Loyo et al., 2007; Lit et al., 2006; Narumi et al., 2000; Wu et al., 2011). Considering the important roles for IFN-γ and IP-10 in the autoimmune process that lead to SLE,

therapeutic neutralization of these key factors could possibly be applied in a clinical setting. Moreover, *M. bovis* mediated induction of lupus in NOD mice could possibly be used as a model for studying the etiopathogenesis of this disease.

In summary, we found the pathogenic inflammatory responses leading to autoimmune diabetes to be multifactorial, and that they depended on a combination of molecular, cellular and stochastic elements. Our findings in TRAIL deficient NOD mice and in NOD mice with low FASL expression revealed novel and intriguing data on the role of TNF family members in controlling islet susceptibility to immune destruction in autoimmune diabetes. We found that TRAIL and FASL regulated the islet expression of the potent chemokine IP-10 and suppressor protein SOCS1 respectively. Suppression of IP-10 and upregulation of *Socs1* transcription, a strong inhibitor of IFN-γ signalling, was associated with suppressed immune cell infiltration of the pancreas and inhibition of diabetes in NOD mice. This study also revealed exciting roles for the proinflammatory cytokine IFN-γ and IP-10 in modulating NOD diabetes in a BCG-mediated diabetes protection model. Information provided here could help in improving our understanding of the pathogenic mechanisms underlying T1D development, and in rationally designing better prognostic tools for this autoimmune condition.

7.4 Potential implications and applications of this study

Profound understanding of the molecular mechanisms of TRAIL, FASL and IFN-γ-associated inhibition of autoimmune diabetes could identify targets suitable for therapeutic manipulation. Interesting propositions arise from the findings in this study. Data from chapters 4 and 5 suggest that TRAIL and FASL signalling are natural mechanisms by which islets inhibit its infiltration and destruction. TRAIL and FASL ligation is capable of suppressing the highly potent, β-cell-toxic chemoattractant, IP-10, and inhibiting the factors leading to its secretion. Genetic or therapeutic modulation of this pathway may therefore enhance preservation of endogenous β-cell function or protection of grafted islets, providing improved treatment of T1D in patients. Results discussed in chapter 6 indicate that BCG instillation in prediabetic NOD mice protected them from autoimmune diabetes, and that IFN-γ signalling was vital in this model. We found reduced effector T cell ratios in BCG treated NOD mice genetically deficient in IFN-γ or the β-chain of its receptor. It is likely that BCG mediated modulation of autoimmunity in this model was a result of redirected trafficking and increased apoptosis of autoaggressive islet-specific immune cells. The temporal and spatial modulation of chemokines or suppressors of proinflammatory cytokines that induce β-cell damage could represent a therapeutic approach that not only preserve β-cell functions in early-detected autoimmune diabetes, but also abrogate islet damage in an advanced stage of the disease. The above assumption finds support in a recent clinical trial that has reported beneficial effects of BCG vaccination in T1D patients (Faustman

et al., 2012). Better prognosis could be achieved with a combination approach with agents that act at different checkpoints in the disease process. In summary, TRAIL, FASL and IFN-γ-associated inhibition of autoimmune diabetes should identify potential avenues of therapeutic intervention. These could include chemoattractants (IP-10), involved in intensifying insulitis, suppressor proteins (SOCS1), potent inhibitor of cytokines that destroy islets, and islet-associated TRAIL/FASL-related molecule, which when ligated, suppress insulitis. Our findings suggest that combining functional aspects of FASL/TRAIL-signalling and BCG-induced mechanisms could induce a robust and stable β-cell–specific immune tolerance, thereby providing protection and improved treatment of autoimmune diabetes in patients.

7.4.1 Mutant mice with natural mechanisms of resistance against T1D could show us new ways of gene regulation.

While screening the genome of an inbred NOD line, a member of the laboratory, Tatiana Toutsman, incidentally identified a highly mutable simple sequence repeat carrying consensus DNA sequences for GATA1 and GATA3-transcription-factor-binding at *D1Bax208* locus in NOD mice. Since *D1Bax208* locus was located immediately 3' to *Fasl*, we examined FASL expression in mice carrying the mutant alleles. Interestingly, these mice had allelic FASL expression, suggesting a GATA transcription-factor-mediated modulation of FASL-expression. Our finding is in accordance with the recent evidence that illustrate GATA

transcription-factor mediated regulation of FASL-expression and an associated variation in biological activity in humans (Tai et al., 2013; Wei et al., 2011). Further experiments with NOD mice carrying the mutant alleles of *D1Bax208* indicated that allelic FASL expression affects the rate of spontaneous diabetes. NOD mice carrying the low-FASL-expressing allele had increased disease incidence, suggesting that modulation of death ligand expression could have a protective effect in NOD diabetes. A profound insight into the molecular biology of GATA-3 binding regions of *Fasl* in NOD lines carrying the mutant *D1Bax208* allele could potentially unravel so far unidentified roles of this death ligand in regulating T1D or even other forms of autoimmunity.

.....

BIBLIOGRAPHY

.....

Abdel-Dayem, H.M., W.S. Omar, M. Aziz, V. Labombardi, L. Difabrizio, J.S. Kempf, and J. Gillooley. 1996. Disseminated mycobacterium avium complex. Review of Ga-67 and TI-201 scans and autopsy findings. *Clinical nuclear medicine* 21:547-556.

Adachi, M., S. Suematsu, T. Suda, D. Watanabe, H. Fukuyama, J. Ogasawara, T. Tanaka, N. Yoshida, and S. Nagata. 1996. Enhanced and accelerated lymphoproliferation in Fas-null mice. *Proc Natl Acad Sci U S A* 93:2131-2136.

Adachi, M., R. Watanabe-Fukunaga, and S. Nagata. 1993. Aberrant transcription caused by the insertion of an early transposable element in an intron of the Fas antigen gene of lpr mice. *Proc Natl Acad Sci U S A* 90:1756-1760.

Akhtar, I., J.P. Gold, L.Y. Pan, J.L. Ferrara, X.D. Yang, J.I. Kim, and K.N. Tan. 1995. CD4+ beta islet cell-reactive T cell clones that suppress autoimmune diabetes in nonobese diabetic mice. *J Exp Med* 182:87-97.

Aktas, O., A. Smorodchenko, S. Brocke, C. Infante-Duarte, U. Schulze Topphoff, J. Vogt, T. Prozorovski, S. Meier, V. Osmanova, E. Pohl, I. Bechmann, R. Nitsch, and F. Zipp. 2005. Neuronal damage in autoimmune neuroinflammation mediated by the death ligand TRAIL. *Neuron* 46:421-432.

Alexander, W.S. 2002. Suppressors of cytokine signalling (SOCS) in the immune system. *Nature reviews. Immunology* 2:410-416.

Alexander, W.S., R. Starr, J.E. Fenner, C.L. Scott, E. Handman, N.S. Sprigg, J.E. Corbin, A.L. Cornish, R. Darwiche, C.M. Owczarek, T.W. Kay, N.A. Nicola, P.J. Hertzog, D. Metcalf, and D.J. Hilton. 1999. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* 98:597-608.

Allen, H.F., G.J. Klingensmith, P. Jensen, E. Simoes, A. Hayward, and H.P. Chase. 1999. Effect of Bacillus Calmette-Guerin vaccination on new-onset type 1 diabetes. A randomized clinical study. *Diabetes care* 22:1703-1707.

Allen, R.D., J.D. Marshall, J.B. Roths, and C.L. Sidman. 1990. Differences defined by bone marrow transplantation suggest that lpr and gld are mutations of genes encoding an interacting pair of molecules. *J Exp Med* 172:1367-1375.

Allison, A.C., A.M. Denman, and R.D. Barnes. 1971. Cooperating and controlling functions of thymus-derived lymphocytes in relation to autoimmunity. *Lancet* 2:135-140.

Allison, J., H.M. Georgiou, A. Strasser, and D.L. Vaux. 1997. Transgenic expression of CD95 ligand on islet beta cells induces a granulocytic infiltration but does not confer immune privilege upon islet allografts. *Proc Natl Acad Sci U S A* 94:3943-3947.

Allison, J., and A. Strasser. 1998. Mechanisms of beta cell death in diabetes: a minor role for CD95. *Proc Natl Acad Sci U S A* 95:13818-13822.

Allison, J., H.E. Thomas, T. Catterall, T.W. Kay, and A. Strasser. 2005. Transgenic expression of dominant-negative Fas-associated death domain protein in beta cells protects against Fas ligand-induced apoptosis and reduces spontaneous diabetes in nonobese diabetic mice. *J Immunol* 175:293-301.

Alyanakian, M.A., F. Grela, A. Aumeunier, C. Chiavaroli, C. Gouarin, E. Bardel, G. Normier, L. Chatenoud, N. Thieblemont, and J.F. Bach. 2006. Transforming growth factor-beta and natural killer T-cells are involved in the protective effect of a bacterial extract on type 1 diabetes. *Diabetes* 55:179-185.

Amedei, A., M.P. Bergman, B.J. Appelmelk, A. Azzurri, M. Benagiano, C. Tamburini, R. van der Zee, J.L. Telford, C.M. Vandenbroucke-Grauls, M.M. D'Elios, and G. Del Prete. 2003. Molecular mimicry between Helicobacter pylori antigens and H+, K+ --adenosine triphosphatase in human gastric autoimmunity. *J Exp Med* 198:1147-1156.

Amrani, A., J. Verdaguer, B. Anderson, T. Utsugi, S. Bou, and P. Santamaria. 1999. Perforin-independent beta-cell destruction by diabetogenic CD8(+) T lymphocytes in transgenic nonobese diabetic mice. *J Clin Invest* 103:1201-1209.

Amrani, A., J. Verdaguer, S. Thiessen, S. Bou, and P. Santamaria. 2000. IL-1alpha, IL-1beta, and IFN-gamma mark beta cells for Fas-dependent destruction by diabetogenic CD4(+) T lymphocytes. *J Clin Invest* 105:459-468.

Anderson, M.K., G. Hernandez-Hoyos, C.J. Dionne, A.M. Arias, D. Chen, and E.V. Rothenberg. 2002. Definition of regulatory network elements for T cell development by perturbation analysis with PU.1 and GATA-3. *Developmental biology* 246:103-121.

Anderson, M.S., and J.A. Bluestone. 2005. The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 23:447-485.

Andre, I., A. Gonzalez, B. Wang, J. Katz, C. Benoist, and D. Mathis. 1996. Checkpoints in the progression of autoimmune disease: lessons from diabetes models. *Proc Natl Acad Sci U S A* 93:2260-2263.

Andrews, B.S., R.A. Eisenberg, A.N. Theofilopoulos, S. Izui, C.B. Wilson, P.J. McConahey, E.D. Murphy, J.B. Roths, and F.J. Dixon. 1978. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *J Exp Med* 148:1198-1215.

Apostolou, I., Z. Hao, K. Rajewsky, and H. von Boehmer. 2003. Effective destruction of Fas-deficient insulin-producing beta cells in type 1 diabetes. *J Exp Med* 198:1103-1106.

Appelmelk, B.J., G. Faller, D. Claeys, T. Kirchner, and C.M. Vandenbroucke-Grauls. 1998. Bugs on trial: the case of Helicobacter pylori and autoimmunity. *Immunol Today* 19:296-299.

Arase, H., N. Arase, Y. Kobayashi, Y. Nishimura, S. Yonehara, and K. Onoe. 1994. Cytotoxicity of fresh NK1.1+ T cell receptor alpha/beta+ thymocytes against a CD4+8+ thymocyte population associated with intact Fas antigen expression on the target. *J Exp Med* 180:423-432.

Argiles, J.M., J. Lopez-Soriano, and F.J. Lopez-Soriano. 1994. Cytokines and diabetes: the final step? Involvement of TNF-alpha in both type I and II diabetes mellitus. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme* 26:447-449.

Ashwell, J.D., R.E. Cunningham, P.D. Noguchi, and D. Hernandez. 1987. Cell growth cycle block of T cell hybridomas upon activation with antigen. *J Exp Med* 165:173-194.

Baboonian, C., M.J. Davies, J.C. Booth, and W.J. McKenna. 1997. Coxsackie B viruses and human heart disease. *Curr Top Microbiol Immunol* 223:31-52.

Baess, I., and B. Mansa. 1978. Determination of genome size and base ratio on deoxyribonucleic acid from mycobacteria. *Acta pathologica et microbiologica Scandinavica. Section B, Microbiology* 86B:309-312.

Bailey, R., J.D. Cooper, L. Zeitels, D.J. Smyth, J.H. Yang, N.M. Walker, E. Hypponen, D.B. Dunger, E. Ramos-Lopez, K. Badenhoop, S. Nejentsev, and J.A. Todd.

2007. Association of the vitamin D metabolism gene CYP27B1 with type 1 diabetes. *Diabetes* 56:2616-2621.

Balomenos, D., R. Rumold, and A.N. Theofilopoulos. 1998. Interferon-gamma is required for lupus-like disease and lymphoaccumulation in MRL-lpr mice. *J Clin Invest* 101:364-371.

Barker, R.N., K.M. Casswell, and C.J. Elson. 1993a. Identification of murine erythrocyte autoantigens and cross-reactive rat antigens. *Immunology* 78:568-573.

Barker, R.N., G.G. de Sa Oliveira, C.J. Elson, and P.M. Lydyard. 1993b. Pathogenic autoantibodies in the NZB mouse are specific for erythrocyte band 3 protein. *Eur J Immunol* 23:1723-1726.

Baron, J.L., E.P. Reich, I. Visintin, and C.A. Janeway, Jr. 1994. The pathogenesis of adoptive murine autoimmune diabetes requires an interaction between alpha 4-integrins and vascular cell adhesion molecule-1. *J Clin Invest* 93:1700-1708.

Barral, A.M., H.E. Thomas, E.M. Ling, R. Darwiche, E. Rodrigo, U. Christen, M. Ejrnaes, T. Wolfe, T.W. Kay, and M.G. von Herrath. 2006. SOCS-1 protects from virally-induced CD8 T cell mediated type 1 diabetes. *Journal of autoimmunity* 27:166-173.

Barrow, W.W., and P.J. Brennan. 1982. Isolation in high frequency of rough variants of Mycobacterium intracellulare lacking C-mycoside glycopeptidolipid antigens. *Journal of bacteriology* 150:381-384.

Baxter, A.G. 2007. The origin and application of experimental autoimmune encephalomyelitis. *Nature reviews. Immunology* 7:904-912.

Baxter, A.G., A.C. Horsfall, D. Healey, P. Ozegbe, S. Day, D.G. Williams, and A. Cooke. 1994. Mycobacteria precipitate an SLE-like syndrome in diabetes-prone NOD mice. *Immunology* 83:227-231.

Baxter, A.G., S.J. Kinder, K.J. Hammond, R. Scollay, and D.I. Godfrey. 1997. Association between alphabetaTCR+CD4-CD8- T-cell deficiency and IDDM in NOD/Lt mice. *Diabetes* 46:572-582.

Baxter, A.G., and T.E. Mandel. 1991. Hemolytic anemia in non-obese diabetic mice. *Eur J Immunol* 21:2051-2055.

Beatty, W.L., E.R. Rhoades, H.J. Ullrich, D. Chatterjee, J.E. Heuser, and D.G. Russell. 2000. Trafficking and release of mycobacterial lipids from infected macrophages. *Traffic* 1:235-247.

Beilke, J.N., C.T. Meagher, K. Hosiawa, M. Champsaur, J.A. Bluestone, and L.L. Lanier. 2012. NK cells are not required for spontaneous autoimmune diabetes in NOD mice. *PloS one* 7:e36011.

Bell, G.I., S. Horita, and J.H. Karam. 1984. A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. *Diabetes* 33:176-183.

Ben-Nun, A., I. Mendel, G. Sappler, and N. Kerlero de Rosbo. 1995. A 12-kDa protein of Mycobacterium tuberculosis protects mice against experimental autoimmune encephalomyelitis. Protection in the absence of shared T cell epitopes with encephalitogenic proteins. *J Immunol* 154:2939-2948.

Bendelac, A., C. Carnaud, C. Boitard, and J.F. Bach. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. *J Exp Med* 166:823-832.

Bendelac, A., O. Lantz, M.E. Quimby, J.W. Yewdell, J.R. Bennink, and R.R. Brutkiewicz. 1995. CD1 recognition by mouse NK1+ T lymphocytes. *Science* 268:863-865.

Bending, D., H. De la Pena, M. Veldhoen, J.M. Phillips, C. Uyttenhove, B. Stockinger, and A. Cooke. 2009. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. *J Clin Invest* 119:565-572.

Bennett, C.L., R. Yoshioka, H. Kiyosawa, D.F. Barker, P.R. Fain, A.O. Shigeoka, and P.F. Chance. 2000. X-Linked syndrome of polyendocrinopathy, immune dysfunction, and diarrhea maps to Xp11.23-Xq13.3. *Am J Hum Genet* 66:461-468.

Bennett, S.T., A.M. Lucassen, S.C. Gough, E.E. Powell, D.E. Undlien, L.E. Pritchard, M.E. Merriman, Y. Kawaguchi, M.J. Dronsfield, F. Pociot, and et al. 1995. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. *Nat Genet* 9:284-292.

Bermudez, L.E. 1994. Immunobiology of Mycobacterium avium infection. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 13:1000-1006.

Berney, T., T. Shibata, R. Merino, Y. Chicheportiche, V. Kindler, P. Vassalli, and S. Izui. 1992. Murine autoimmune hemolytic anemia resulting from Fc gamma receptor-mediated erythrophagocytosis: protection by erythropoietin but not by interleukin-3, and aggravation by granulocyte-macrophage colony-stimulating factor. *Blood* 79:2960-2964.

Beutler, B., and C. van Huffel. 1994. Unraveling function in the TNF ligand and receptor families. *Science* 264:667-668.

Bielschowsky, M., and F. Bielschowsky. 1964. Observations on Nzb/B1 Mice; Differential Fertility in Reciprocal Crosses and the Transmission of the Auto-Immune Haemolytic Anaemia to Nzb/B1 X Nzc/B1 Hybrids. *The Australian journal of experimental biology and medical science* 42:561-568.

Bielschowsky, M., B.J. Helyer, and J.B. Howie. 1959. *Proc. Univ. Otago Med. School* 37:

Biron, C.A., K.B. Nguyen, G.C. Pien, L.P. Cousens, and T.P. Salazar-Mather. 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. *Annu Rev Immunol* 17:189-220.

Bjornvold, M., M.C. Munthe-Kaas, T. Egeland, G. Joner, K. Dahl-Jorgensen, P.R. Njolstad, H.E. Akselsen, K. Gervin, K.C. Carlsen, K.H. Carlsen, and D.E. Undlien. 2009. A TLR2 polymorphism is associated with type 1 diabetes and allergic asthma. *Genes and immunity* 10:181-187.

Block, S.R., J.B. Winfield, M.D. Lockshin, W.A. D'Angelo, and C.L. Christian. 1975. Studies of twins with systemic lupus erythematosus. A review of the literature and presentation of 12 additional sets. *Am J Med* 59:533-552.

Boehm, U., T. Klamp, M. Groot, and J.C. Howard. 1997. Cellular responses to interferon-gamma. *Annu Rev Immunol* 15:749-795.

Bosque, A., J.I. Aguilo, M. del Rey, E. Paz-Artal, L.M. Allende, J. Naval, and A. Anel. 2008. Cell cycle regulation by FasL and Apo2L/TRAIL in human T-cell blasts. Implications for autoimmune lymphoproliferative syndromes. *Journal of leukocyte biology* 84:488-498.

Bossu, P., G.G. Singer, P. Andres, R. Ettinger, A. Marshak-Rothstein, and A.K. Abbas. 1993. Mature CD4+ T lymphocytes from MRL/lpr mice are resistant to receptor-mediated tolerance and apoptosis. *J Immunol* 151:7233-7239.

Bouralexis, S., D.M. Findlay, and A. Evdokiou. 2005. Death to the bad guys: targeting cancer via Apo2L/TRAIL. *Apoptosis : an international journal on programmed cell death* 10:35-51.

Bradley, L.M., V.C. Asensio, L.K. Schioetz, J. Harbertson, T. Krahl, G. Patstone, N. Woolf, I.L. Campbell, and N. Sarvetnick. 1999. Islet-specific Th1, but not Th2, cells secrete multiple chemokines and promote rapid induction of autoimmune diabetes. *J Immunol* 162:2511-2520.

Brauner, H., M. Elemans, S. Lemos, C. Broberger, D. Holmberg, M. Flodstrom-Tullberg, K. Karre, and P. Hoglund. 2010. Distinct phenotype and function of NK cells in the pancreas of nonobese diabetic mice. *J Immunol* 184:2272-2280.

Brenden, N., and J. Bohme. 1999. Differential MHC expression requirements for positive selection of separate TCR Vb families. *Immunogenetics* 49:1-6.

Broderick, T.L., M. Jankowski, D. Wang, B.A. Danalache, C.R. Parrott, and J. Gutkowska. 2012. Downregulation in GATA4 and Downstream Structural and Contractile Genes in the db/db Mouse Heart. *ISRN endocrinology* 2012:736860.

Brossart, P., and M.J. Bevan. 1996. Selective activation of Fas/Fas ligand-mediated cytotoxicity by a self peptide. *J Exp Med* 183:2449-2458.

Brunner, T., R.J. Mogil, D. LaFace, N.J. Yoo, A. Mahboubi, F. Echeverri, S.J. Martin, W.R. Force, D.H. Lynch, C.F. Ware, and et al. 1995. Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. *Nature* 373:441-444.

Brusko, T.M., C.H. Wasserfall, M.J. Clare-Salzler, D.A. Schatz, and M.A. Atkinson. 2005. Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. *Diabetes* 54:1407-1414.

Calmette, A., and C. Guérin. 1911. *Ann. Inst. Pasteur* 25:

Calmette, A., and C. Guérin. 1920. *Ann. Inst. Pasteur* 34:

Campbell, I.L., T.W. Kay, L. Oxbrow, and L.C. Harrison. 1991. Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J Clin Invest* 87:739-742.

Cao, W., S.S. Tykodi, M.T. Esser, V.L. Braciale, and T.J. Braciale. 1995. Partial activation of CD8+ T cells by a self-derived peptide. *Nature* 378:295-298.

Cardozo, A.K., H. Heimberg, Y. Heremans, R. Leeman, B. Kutlu, M. Kruhoffer, T. Orntoft, and D.L. Eizirik. 2001a. A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-dependent genes in primary rat pancreatic beta-cells. *The Journal of biological chemistry* 276:48879-48886.

Cardozo, A.K., M. Kruhoffer, R. Leeman, T. Orntoft, and D.L. Eizirik. 2001b. Identification of novel cytokine-induced genes in pancreatic beta-cells by high-density oligonucleotide arrays. *Diabetes* 50:909-920.

Cardozo, A.K., P. Proost, C. Gysemans, M.C. Chen, C. Mathieu, and D.L. Eizirik. 2003. IL-1beta and IFN-gamma induce the expression of diverse chemokines and IL-15 in human and rat pancreatic islet cells, and in islets from pre-diabetic NOD mice. *Diabetologia* 46:255-266.

Carnaud, C., J. Gombert, O. Donnars, H. Garchon, and A. Herbelin. 2001. Protection against diabetes and improved NK/NKT cell performance in NOD.NK1.1 mice congenic at the NK complex. *J Immunol* 166:2404-2411.

Carvalho-Pinto, C., M.I. Garcia, L. Gomez, A. Ballesteros, A. Zaballos, J.M. Flores, M. Mellado, J.M. Rodriguez-Frade, D. Balomenos, and A.C. Martinez. 2004. Leukocyte attraction through the CCR5 receptor controls progress from insulitis to diabetes in non-obese diabetic mice. *Eur J Immunol* 34:548-557.

Cermelli, C., R. Berti, S.S. Soldan, M. Mayne, M. D'Ambrosia J, S.K. Ludwin, and S. Jacobson. 2003. High frequency of human herpesvirus 6 DNA in multiple sclerosis plaques isolated by laser microdissection. *J Infect Dis* 187:1377-1387.

Chang, J.H., Y.J. Kim, S.H. Han, and C.Y. Kang. 2009. IFN-gamma-STAT1 signal regulates the differentiation of inducible Treg: potential role for ROS-mediated apoptosis. *Eur J Immunol* 39:1241-1251.

Chase, H.P., T.A. MacKenzie, J. Burdick, R. Fiallo-Scharer, P. Walravens, G. Klingensmith, and M. Rewers. 2004. Redefining the clinical remission period in children with type 1 diabetes. *Pediatr Diabetes* 5:16-19.

Chee, D.O., and A.J. Bodurtha. 1974. Facilitation and inhibition of b16 melanoma by BCG in vivo and by lymphoid cells from bcg-treated mice in vitro. *International journal of cancer. Journal international du cancer* 14:137-143.

Chen, H.H., F.Y. Tsai, and C.T. Chen. 2001. Negative regulatory regions of the PAT1 promoter of Hz-1 virus contain GATA elements which associate with cellular factors and regulate promoter activity. *The Journal of general virology* 82:313-320.

Chen, Y.G., C.M. Choisy-Rossi, T.M. Holl, H.D. Chapman, G.S. Besra, S.A. Porcelli, D.J. Shaffer, D. Roopenian, S.B. Wilson, and D.V. Serreze. 2005. Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. *J Immunol* 174:1196-1204.

Cheng, Y., W. Wu, S.A. Kumar, D. Yu, W. Deng, T. Tripic, D.C. King, K.B. Chen, Y. Zhang, D. Drautz, B. Giardine, S.C. Schuster, W. Miller, F. Chiaromonte, Y. Zhang, G.A. Blobel, M.J. Weiss, and R.C. Hardison. 2009. Erythroid GATA1 function revealed by genome-wide analysis of transcription factor occupancy, histone modifications, and mRNA expression. *Genome research* 19:2172-2184.

Chervonsky, A.V., Y. Wang, F.S. Wong, I. Visintin, R.A. Flavell, C.A. Janeway, Jr., and L.A. Matis. 1997. The role of Fas in autoimmune diabetes. *Cell* 89:17-24.

Cheung, S.S., D.L. Metzger, X. Wang, J. Huang, J. Tai, A.J. Tingle, and D. Ou. 2005. Tumor necrosis factor-related apoptosis-inducing ligand and CD56 expression in patients with type 1 diabetes mellitus. *Pancreas* 30:105-114.

Chiba, A., C.C. Dascher, G.S. Besra, and M.B. Brenner. 2008. Rapid NKT cell responses are self-terminating during the course of microbial infection. *J Immunol* 181:2292-2302.

Chin, K., N. Oda, K. Shen, and C.T. Noguchi. 1995. Regulation of transcription of the human erythropoietin receptor gene by proteins binding to GATA-1 and Sp1 motifs. *Nucleic acids research* 23:3041-3049.

Chong, A.S., J. Shen, J. Tao, D. Yin, A. Kuznetsov, M. Hara, and L.H. Philipson. 2006. Reversal of diabetes in non-obese diabetic mice without spleen cell-derived beta cell regeneration. *Science* 311:1774-1775.

Chong, M.M., Y. Chen, R. Darwiche, N.L. Dudek, W. Irawaty, P. Santamaria, J. Allison, T.W. Kay, and H.E. Thomas. 2004. Suppressor of cytokine signaling-1

overexpression protects pancreatic beta cells from CD8+ T cell-mediated autoimmune destruction. *J Immunol* 172:5714-5721.

Chong, M.M., H.E. Thomas, and T.W. Kay. 2002. Suppressor of cytokine signaling-1 regulates the sensitivity of pancreatic beta cells to tumor necrosis factor. *The Journal of biological chemistry* 277:27945-27952.

Christen, U., D. Benke, T. Wolfe, E. Rodrigo, A. Rhode, A.C. Hughes, M.B. Oldstone, and M.G. von Herrath. 2004. Cure of prediabetic mice by viral infections involves lymphocyte recruitment along an IP-10 gradient. *J Clin Invest* 113:74-84.

Christen, U., T. Wolfe, U. Mohrle, A.C. Hughes, E. Rodrigo, E.A. Green, R.A. Flavell, and M.G. von Herrath. 2001. A dual role for TNF-alpha in type 1 diabetes: islet-specific expression abrogates the ongoing autoimmune process when induced late but not early during pathogenesis. *J Immunol* 166:7023-7032.

Chu, J.L., P. Ramos, A. Rosendorff, J. Nikolic-Zugic, E. Lacy, A. Matsuzawa, and K.B. Elkon. 1995. Massive upregulation of the Fas ligand in lpr and gld mice: implications for Fas regulation and the graft-versus-host disease-like wasting syndrome. *J Exp Med* 181:393-398.

Clare-Salzler, M.J., J. Brooks, A. Chai, K. Van Herle, and C. Anderson. 1992. Prevention of diabetes in nonobese diabetic mice by dendritic cell transfer. *J Clin Invest* 90:741-748.

Cohen, P.L., and R.A. Eisenberg. 1991. Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. *Annu Rev Immunol* 9:243-269.

Cooper, A.M., D.K. Dalton, T.A. Stewart, J.P. Griffin, D.G. Russell, and I.M. Orme. 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med* 178:2243-2247.

Cooper, G.S., M.L. Bynum, and E.C. Somers. 2009a. Recent insights in the epidemiology of autoimmune diseases: improved prevalence estimates and understanding of clustering of diseases. *Journal of autoimmunity* 33:197-207.

Cooper, J.D., D.J. Smyth, A.M. Smiles, V. Plagnol, N.M. Walker, J.E. Allen, K. Downes, J.C. Barrett, B.C. Healy, J.C. Mychaleckyj, J.H. Warram, and J.A. Todd. 2008.

Meta-analysis of genome-wide association study data identifies additional type 1 diabetes risk loci. *Nat Genet* 40:1399-1401.

Cooper, J.D., D.J. Smyth, N.M. Walker, H. Stevens, O.S. Burren, C. Wallace, C. Greissl, E. Ramos-Lopez, E. Hypponen, D.B. Dunger, T.D. Spector, W.H. Ouwehand, T.J. Wang, K. Badenhoop, and J.A. Todd. 2011. Inherited variation in vitamin D genes is associated with predisposition to autoimmune disease type 1 diabetes. *Diabetes* 60:1624-1631.

Cooper, J.D., N.M. Walker, D.J. Smyth, K. Downes, B.C. Healy, J.A. Todd, and I.D.G.C. Type. 2009b. Follow-up of 1715 SNPs from the Wellcome Trust Case Control Consortium genome-wide association study in type I diabetes families. *Genes and immunity* 10 Suppl 1:S85-94.

Coppieters, K.T., F. Dotta, N. Amirian, P.D. Campbell, T.W. Kay, M.A. Atkinson, B.O. Roep, and M.G. von Herrath. 2012. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. *J Exp Med* 209:51-60.

Corazza, N., G. Brumatti, S. Jakob, A. Villunger, and T. Brunner. 2004. TRAIL and thymocyte apoptosis: not so deadly? *Cell death and differentiation* 11 Suppl 2:S213-215.

Cottet, S., P. Dupraz, F. Hamburger, W. Dolci, M. Jaquet, and B. Thorens. 2001. SOCS-1 protein prevents Janus Kinase/STAT-dependent inhibition of beta cell insulin gene transcription and secretion in response to interferon-gamma. *The Journal of biological chemistry* 276:25862-25870.

Cox, K.O., and D. Keast. 1974. Autoimmune haemolytic anaemia induced in mice immunized with rat erythrocytes. *Clinical and experimental immunology* 17:319-327.

Cretney, E., J.L. McQualter, N. Kayagaki, H. Yagita, C.C. Bernard, I.S. Grewal, A. Ashkenazi, and M.J. Smyth. 2005. TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L suppresses experimental autoimmune encephalomyelitis in mice. *Immunology and cell biology* 83:511-519.

Cretney, E., A.P. Uldrich, S.P. Berzins, A. Strasser, D.I. Godfrey, and M.J. Smyth. 2003. Normal thymocyte negative selection in TRAIL-deficient mice. *J Exp Med* 198:491-496.

Cruz, A., S.A. Khader, E. Torrado, A. Fraga, J.E. Pearl, J. Pedrosa, A.M. Cooper, and A.G. Castro. 2006. Cutting edge: IFN-gamma regulates the induction and expansion of IL-17-producing CD4 T cells during mycobacterial infection. *J Immunol* 177:1416-1420.

Dacie, J.V. 1962. The haemolytic anemias. Part II: The autoimmune haemolytic anemias. *Churchill London 2nd ed* 341:

Dacie, J.V., and S.M. Worlledge. 1969. Auto-immune hemolytic anemias. *Progress in hematology* 6:82-120.

Dahlen, E., K. Dawe, L. Ohlsson, and G. Hedlund. 1998. Dendritic cells and macrophages are the first and major producers of TNF-alpha in pancreatic islets in the nonobese diabetic mouse. *J Immunol* 160:3585-3593.

Danaei, G., M.M. Finucane, Y. Lu, G.M. Singh, M.J. Cowan, C.J. Paciorek, J.K. Lin, F. Farzadfar, Y.H. Khang, G.A. Stevens, M. Rao, M.K. Ali, L.M. Riley, C.A. Robinson, M. Ezzati, and G. Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating. 2011. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet* 378:31-40.

Daniel, P.T., and P.H. Krammer. 1994. Activation induces sensitivity toward APO-1 (CD95)-mediated apoptosis in human B cells. *J Immunol* 152:5624-5632.

David-Watine, B., A. Israel, and P. Kourilsky. 1990. The regulation and expression of MHC class I genes. *Immunol Today* 11:286-292.

de la Barrera, S., M. Finiasz, S. Fink, J. Ilarregui, M. Aleman, L. Olivares, M.C. Franco, G. Pizzariello, and M. del Carmen Sasiain. 2004. NK cells modulate the cytotoxic activity generated by Mycobacterium leprae-hsp65 in leprosy patients: role of IL-18 and IL-13. *Clinical and experimental immunology* 135:105-113.

Debray-Sachs, M., C. Carnaud, C. Boitard, H. Cohen, I. Gresser, P. Bedossa, and J.F. Bach. 1991. Prevention of diabetes in NOD mice treated with antibody to murine IFN gamma. *Journal of autoimmunity* 4:237-248.

Degli-Esposti, M.A., P.J. Smolak, H. Walczak, J. Waugh, C.P. Huang, R.F. DuBose, R.G. Goodwin, and C.A. Smith. 1997. Cloning and characterization of TRAIL-R3, a

novel member of the emerging TRAIL receptor family. *J Exp Med* 186:1165-1170.

Del-Rey, M., J. Ruiz-Contreras, A. Bosque, S. Calleja, J. Gomez-Rial, E. Roldan, P. Morales, A. Serrano, A. Anel, E. Paz-Artal, and L.M. Allende. 2006. A homozygous Fas ligand gene mutation in a patient causes a new type of autoimmune lymphoproliferative syndrome. *Blood* 108:1306-1312.

Desbarats, J., R.C. Duke, and M.K. Newell. 1998. Newly discovered role for Fas ligand in the cell-cycle arrest of CD4+ T cells. *Nat Med* 4:1377-1382.

Devendra, D., and G.S. Eisenbarth. 2003. 17. Immunologic endocrine disorders. *The Journal of allergy and clinical immunology* 111:S624-636.

Dharnidharka, V.R., Y. Van Patten, F.R. Bahjat, and M. Clare-Salzler. 2002. Fas stimulation results in selective islet infiltrate apoptosis in situ and reversal of diabetes. *Annals of the New York Academy of Sciences* 958:160-162.

Dhein, J., H. Walczak, C. Baumler, K.M. Debatin, and P.H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature* 373:438-441.

DIAMOND. 2006. DIAMOND Project Group: Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999. *Diabet Med* 23:857-866.

Diehl, G.E., H.H. Yue, K. Hsieh, A.A. Kuang, M. Ho, L.A. Morici, L.L. Lenz, D. Cado, L.W. Riley, and A. Winoto. 2004. TRAIL-R as a negative regulator of innate immune cell responses. *Immunity* 21:877-889.

Dotta, F., S. Censini, A.G. van Halteren, L. Marselli, M. Masini, S. Dionisi, F. Mosca, U. Boggi, A.O. Muda, S.D. Prato, J.F. Elliott, A. Covacci, R. Rappuoli, B.O. Roep, and P. Marchetti. 2007. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. *Proc Natl Acad Sci U S A* 104:5115-5120.

Draper, P. 1974. The mycoside capsule of Mycobacterium Avium 357. *Journal of general microbiology* 83:431-433.

Dwyer, J.M., and C. Johnson. 1981. The use of concanavalin A to study the immunoregulation of human T cells. *Clinical and experimental immunology* 46:237-249.

Eddy, S., R. Wim, V.E. Peter, R. Tanja, T. Jan, and V.S. Werner. 1999. Myasthenia gravis: another autoimmune disease associated with hepatitis C virus infection. *Dig Dis Sci* 44:186-189.

Eisenberg, R.A., S.Y. Craven, R.W. Warren, and P.L. Cohen. 1987. Stochastic control of anti-Sm autoantibodies in MRL/Mp-lpr/lpr mice. *J Clin Invest* 80:691-697.

Eizirik, D.L., M.L. Colli, and F. Ortis. 2009. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. *Nature reviews. Endocrinology* 5:219-226.

Eizirik, D.L., M. Flodstrom, A.E. Karlsen, and N. Welsh. 1996. The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* 39:875-890.

Elliott, J.F., K.L. Marlin, and R.M. Couch. 1998. Effect of bacille Calmette-Guerin vaccination on C-peptide secretion in children newly diagnosed with IDDM. *Diabetes care* 21:1691-1693.

Emamaullee, J.A., J. Davis, S. Merani, C. Toso, J.F. Elliott, A. Thiesen, and A.M. Shapiro. 2009. Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice. *Diabetes* 58:1302-1311.

Ermann, J., and C.G. Fathman. 2001. Autoimmune diseases: genes, bugs and failed regulation. *Nat Immunol* 2:759-761.

Esser, M.T., B. Krishnamurthy, and V.L. Braciale. 1996. Distinct T cell receptor signaling requirements for perforin- or FasL-mediated cytotoxicity. *J Exp Med* 183:1697-1706.

EURODIAB. 2000. Infections and vaccinations as risk factors for childhood type I (insulin-dependent) diabetes mellitus: a multicentre case-control investigation. EURODIAB Substudy 2 Study Group. *Diabetologia* 43:47-53.

Fairchild, S., A.M. Knight, P.J. Dyson, and K. Tomonari. 1991. Co-segregation of a gene encoding a deletion ligand for Tcrb-V3+ T cells with Mtv-3. *Immunogenetics* 34:227-230.

Falschlehner, C., U. Schaefer, and H. Walczak. 2009. Following TRAIL's path in the immune system. *Immunology* 127:145-154.

Fanger, N.A., C.R. Maliszewski, K. Schooley, and T.S. Griffith. 1999. Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Exp Med* 190:1155-1164.

Faustman, D.L., L. Wang, Y. Okubo, D. Burger, L. Ban, G. Man, H. Zheng, D. Schoenfeld, R. Pompei, J. Avruch, and D.M. Nathan. 2012. Proof-of-concept,

randomized, controlled clinical trial of Bacillus-Calmette-Guerin for treatment of long-term type 1 diabetes. *PloS one* 7:e41756.

Ferraro, A., C. Socci, A. Stabilini, A. Valle, P. Monti, L. Piemonti, R. Nano, S. Olek, P. Maffi, M. Scavini, A. Secchi, C. Staudacher, E. Bonifacio, and M. Battaglia. 2011. Expansion of Th17 cells and functional defects in T regulatory cells are key features of the pancreatic lymph nodes in patients with type 1 diabetes. *Diabetes* 60:2903-2913.

Ferri, C., D. Giuggioli, M. Cazzato, M. Sebastiani, M.T. Mascia, and A.L. Zignego. 2003. HCV-related cryoglobulinemic vasculitis: an update on its etiopathogenesis and therapeutic strategies. *Clin Exp Rheumatol* 21:S78-84.

Feuerer, M., Y. Shen, D.R. Littman, C. Benoist, and D. Mathis. 2009. How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. *Immunity* 31:654-664.

Filippi, C.M., E.A. Estes, J.E. Oldham, and M.G. von Herrath. 2009. Immunoregulatory mechanisms triggered by viral infections protect from type 1 diabetes in mice. *J Clin Invest* 119:1515-1523.

Fisher, G.H., F.J. Rosenberg, S.E. Straus, J.K. Dale, L.A. Middleton, A.Y. Lin, W. Strober, M.J. Lenardo, and J.M. Puck. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell* 81:935-946.

Flodstrom-Tullberg, M., D. Yadav, R. Hagerkvist, D. Tsai, P. Secrest, A. Stotland, and N. Sarvetnick. 2003. Target cell expression of suppressor of cytokine signaling-1 prevents diabetes in the NOD mouse. *Diabetes* 52:2696-2700.

Flynn, J.L., J. Chan, K.J. Triebold, D.K. Dalton, T.A. Stewart, and B.R. Bloom. 1993. An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med* 178:2249-2254.

Fortner, K.A., and R.C. Budd. 2005. The death receptor Fas (CD95/APO-1) mediates the deletion of T lymphocytes undergoing homeostatic proliferation. *J Immunol* 175:4374-4382.

Fossati-Jimack, L., L. Reininger, Y. Chicheportiche, R. Clynes, J.V. Ravetch, T. Honjo, and S. Izui. 1999. High pathogenic potential of low-affinity autoantibodies in experimental autoimmune hemolytic anemia. *J Exp Med* 190:1689-1696.

Fragoso-Loyo, H., Y. Richaud-Patin, A. Orozco-Narvaez, L. Davila-Maldonado, Y. Atisha-Fregoso, L. Llorente, and J. Sanchez-Guerrero. 2007. Interleukin-6 and chemokines in the neuropsychiatric manifestations of systemic lupus erythematosus. *Arthritis Rheum* 56:1242-1250.

Franke, D.D., E.S. Yolcu, P. Alard, M.M. Kosiewicz, and H. Shirwan. 2007. A novel multimeric form of FasL modulates the ability of diabetogenic T cells to mediate type 1 diabetes in an adoptive transfer model. *Molecular immunology* 44:2884-2892.

Frigerio, S., T. Junt, B. Lu, C. Gerard, U. Zumsteg, G.A. Hollander, and L. Piali. 2002. Beta cells are responsible for CXCR3-mediated T-cell infiltration in insulitis. *Nat Med* 8:1414-1420.

Fujiwara, T., H. O'Geen, S. Keles, K. Blahnik, A.K. Linnemann, Y.A. Kang, K. Choi, P.J. Farnham, and E.H. Bresnick. 2009. Discovering hematopoietic mechanisms through genome-wide analysis of GATA factor chromatin occupancy. *Molecular cell* 36:667-681.

Fung, E.S., C. Thurm, R. Reuille, B. Brede, and B.T. Livingston. 2005. Identification of cis-regulatory elements involved in transcriptional regulation of the sea urchin SpFoxB gene. *Development, growth & differentiation* 47:461-470.

Fuss, I.J., F. Heller, M. Boirivant, F. Leon, M. Yoshida, S. Fichtner-Feigl, Z. Yang, M. Exley, A. Kitani, R.S. Blumberg, P. Mannon, and W. Strober. 2004. Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J Clin Invest* 113:1490-1497.

Gattass, C.R., L.B. King, A.D. Luster, and J.D. Ashwell. 1994. Constitutive expression of interferon gamma-inducible protein 10 in lymphoid organs and inducible expression in T cells and thymocytes. *J Exp Med* 179:1373-1378.

Gaudreau, S., C. Guindi, M. Menard, G. Besin, G. Dupuis, and A. Amrani. 2007. Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of CD4+CD25+ regulatory T cells. *J Immunol* 179:3638-3647.

Gehrs, B.C., and R.C. Friedberg. 2002. Autoimmune hemolytic anemia. *American journal of hematology* 69:258-271.

Ghosh, A., Y. Dogan, M. Moroz, A.M. Holland, N.L. Yim, U.K. Rao, L.F. Young, D. Tannenbaum, D. Masih, E. Velardi, J.J. Tsai, R.R. Jenq, O. Penack, A.M. Hanash, O.M. Smith, K. Piersanti, C. Lezcano, G.F. Murphy, C. Liu, M.L. Palomba, M.G. Sauer, M. Sadelain, V. Ponomarev, and M.R. van den Brink. 2013. Adoptively transferred TRAIL+ T cells suppress GVHD and augment antitumor activity. *J Clin Invest* 123:2654-2662.

Giarratana, N., G. Penna, S. Amuchastegui, R. Mariani, K.C. Daniel, and L. Adorini. 2004. A vitamin D analog down-regulates proinflammatory chemokine production by pancreatic islets inhibiting T cell recruitment and type 1 diabetes development. *J Immunol* 173:2280-2287.

Goldberg, E., and I. Krause. 2009. Infection and type 1 diabetes mellitus - a two edged sword? *Autoimmunity reviews* 8:682-686.

Gray, D.H., I. Gavanescu, C. Benoist, and D. Mathis. 2007. Danger-free autoimmune disease in Aire-deficient mice. *Proc Natl Acad Sci U S A* 104:18193-18198.

Green, E.A., and R.A. Flavell. 1999. The initiation of autoimmune diabetes. *Current opinion in immunology* 11:663-669.

Griffith, T.S., H. Kazama, R.L. VanOosten, J.K. Earle, Jr., J.M. Herndon, D.R. Green, and T.A. Ferguson. 2007. Apoptotic cells induce tolerance by generating helpless CD8+ T cells that produce TRAIL. *J Immunol* 178:2679-2687.

Griffith, T.S., S.R. Wiley, M.Z. Kubin, L.M. Sedger, C.R. Maliszewski, and N.A. Fanger. 1999. Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. *J Exp Med* 189:1343-1354.

Griseri, T., L. Beaudoin, J. Novak, L.T. Mars, F. Lepault, R. Liblau, and A. Lehuen. 2005. Invariant NKT cells exacerbate type 1 diabetes induced by CD8 T cells. *J Immunol* 175:2091-2101.

Gustavsson, C., E. Agardh, B. Bengtsson, and C.D. Agardh. 2008. TNF-alpha is an independent serum marker for proliferative retinopathy in type 1 diabetic patients. *Journal of diabetes and its complications* 22:309-316.

Haas, C., B. Ryffel, and M. Le Hir. 1998. IFN-gamma receptor deletion prevents autoantibody production and glomerulonephritis in lupus-prone (NZB x NZW)F1 mice. *J Immunol* 160:3713-3718.

Halaas, O., R. Vik, A. Ashkenazi, and T. Espevik. 2000. Lipopolysaccharide induces expression of APO2 ligand/TRAIL in human monocytes and macrophages. *Scandinavian journal of immunology* 51:244-250.

Hall, A.M., F.J. Ward, M.A. Vickers, L.M. Stott, S.J. Urbaniak, and R.N. Barker. 2002. Interleukin-10-mediated regulatory T-cell responses to epitopes on a human red blood cell autoantigen. *Blood* 100:4529-4536.

Hammond, K.J., D.G. Pellicci, L.D. Poulton, O.V. Naidenko, A.A. Scalzo, A.G. Baxter, and D.I. Godfrey. 2001. CD1d-restricted NKT cells: an interstrain comparison. *J Immunol* 167:1164-1173.

Harada, M., Y. Kishimoto, and S. Makino. 1990. Prevention of overt diabetes and insulitis in NOD mice by a single BCG vaccination. *Diabetes Res Clin Pract* 8:85-89.

Harigai, M., M. Kawamoto, M. Hara, T. Kubota, N. Kamatani, and N. Miyasaka. 2008. Excessive production of IFN-gamma in patients with systemic lupus erythematosus and its contribution to induction of B lymphocyte stimulator/B cell-activating factor/TNF ligand superfamily-13B. *J Immunol* 181:2211-2219.

Harrington, L.E., R.D. Hatton, P.R. Mangan, H. Turner, T.L. Murphy, K.M. Murphy, and C.T. Weaver. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol* 6:1123-1132.

Hashemi, M., A. Fazaeli, S. Ghavami, E. Eskandari-Nasab, F. Arbabi, M.A. Mashhadi, M. Taheri, W. Chaabane, M.V. Jain, and M.J. Los. 2013. Functional polymorphisms of FAS and FASL gene and risk of breast cancer - pilot study of 134 cases. *PloS one* 8:e53075.

Hattori, M., J.B. Buse, R.A. Jackson, L. Glimcher, M.E. Dorf, M. Minami, S. Makino, K. Moriwaki, H. Kuzuya, H. Imura, and et al. 1986. The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. *Science* 231:733-735.

Hawke, C.G., D.M. Painter, P.D. Kirwan, R.R. Van Driel, and A.G. Baxter. 2003. Mycobacteria, an environmental enhancer of lupus nephritis in a mouse model of systemic lupus erythematosus. *Immunology* 108:70-78.

Heldwein, K.A., M.D. Liang, T.K. Andresen, K.E. Thomas, A.M. Marty, N. Cuesta, S.N. Vogel, and M.J. Fenton. 2003. TLR2 and TLR4 serve distinct roles in the host immune response against Mycobacterium bovis BCG. *Journal of leukocyte biology* 74:277-286.

Helyer, B.J., and J.B. Howie. 1963. Spontaneous auto-immune disease in NZB/BL mice. *British journal of haematology* 9:119-131.

Herbeuval, J.P., A.W. Hardy, A. Boasso, S.A. Anderson, M.J. Dolan, M. Dy, and G.M. Shearer. 2005. Regulation of TNF-related apoptosis-inducing ligand on primary CD4+ T cells by HIV-1: role of type I IFN-producing plasmacytoid dendritic cells. *Proc Natl Acad Sci U S A* 102:13974-13979.

Herbeuval, J.P., C. Lambert, O. Sabido, M. Cottier, P. Fournel, M. Dy, and C. Genin. 2003. Macrophages from cancer patients: analysis of TRAIL, TRAIL receptors, and colon tumor cell apoptosis. *Journal of the National Cancer Institute* 95:611-621.

Herold, K.C., A. Huen, L. Gould, H. Traisman, and A.H. Rubenstein. 1984. Alterations in lymphocyte subpopulations in type 1 (insulin-dependent) diabetes mellitus: exploration of possible mechanisms and relationships to autoimmune phenomena. *Diabetologia* 27 Suppl:102-105.

Higuchi, Y., P. Herrera, P. Muniesa, J. Huarte, D. Belin, P. Ohashi, P. Aichele, L. Orci, J.D. Vassalli, and P. Vassalli. 1992. Expression of a tumor necrosis factor alpha transgene in murine pancreatic beta cells results in severe and permanent insulitis without evolution towards diabetes. *J Exp Med* 176:1719-1731.

Hilliard, B., A. Wilmen, C. Seidel, T.S. Liu, R. Goke, and Y. Chen. 2001. Roles of TNF-related apoptosis-inducing ligand in experimental autoimmune encephalomyelitis. *J Immunol* 166:1314-1319.

Hogan, L.H., W. Markofski, A. Bock, B. Barger, J.D. Morrissey, and M. Sandor. 2001. Mycobacterium bovis BCG-induced granuloma formation depends on gamma interferon and CD40 ligand but does not require CD28. *Infection and immunity* 69:2596-2603.

Holmes, M.C., J. Gorie, and F.M. Burnet. 1961. Transmission by splenic cells of an autoimmune disease occurring spontaneously in mice. *Lancet* 2:638-639.

Honeyman, M.C., B.S. Coulson, N.L. Stone, S.A. Gellert, P.N. Goldwater, C.E. Steele, J.J. Couper, B.D. Tait, P.G. Colman, and L.C. Harrison. 2000. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. *Diabetes* 49:1319-1324.

Honkanen, J., J.K. Nieminen, R. Gao, K. Luopajarvi, H.M. Salo, J. Ilonen, M. Knip, T. Otonkoski, and O. Vaarala. 2010. IL-17 immunity in human type 1 diabetes. *J Immunol* 185:1959-1967.

Horwitz, M.S., L.M. Bradley, J. Harbertson, T. Krahl, J. Lee, and N. Sarvetnick. 1998. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med* 4:781-785.

Hultgren, B., X. Huang, N. Dybdal, and T.A. Stewart. 1996. Genetic absence of gamma-interferon delays but does not prevent diabetes in NOD mice. *Diabetes* 45:812-817.

Humphreys-Beher, M.G., L. Brinkley, K.R. Purushotham, P.L. Wang, Y. Nakagawa, D. Dusek, M. Kerr, N. Chegini, and E.K. Chan. 1993. Characterization of antinuclear autoantibodies present in the serum from nonobese diabetic (NOD) mice. *Clinical immunology and immunopathology* 68:350-356.

IDF. 2011. IDF Diabetes Atlas, 5th edn. Brussels, Belgium: International Diabetes Federation, 2011.

Imagawa, S., M. Yamamoto, and Y. Miura. 1997. Negative regulation of the erythropoietin gene expression by the GATA transcription factors. *Blood* 89:1430-1439.

Ishibashi, T., H. Yamada, S. Harada, Y. Harada, M. Takamoto, and K. Sugiyama. 1978. Inhibition and promotion of tumor growth by BCG: evidence for stimulation of humoral enhancing factors by BCG. *International journal of cancer. Journal international du cancer* 21:67-71.

Ishida, H., T. Muchamuel, S. Sakaguchi, S. Andrade, S. Menon, and M. Howard. 1994. Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F1 mice. *J Exp Med* 179:305-310.

Ishizuka, N., K. Yagui, Y. Tokuyama, K. Yamada, Y. Suzuki, J. Miyazaki, N. Hashimoto, H. Makino, Y. Saito, and A. Kanatsuka. 1999. Tumor necrosis factor alpha signaling pathway and apoptosis in pancreatic beta cells. *Metabolism: clinical and experimental* 48:1485-1492.

Itoh, N., A. Imagawa, T. Hanafusa, M. Waguri, K. Yamamoto, H. Iwahashi, M. Moriwaki, H. Nakajima, J. Miyagawa, M. Namba, S. Makino, S. Nagata, N. Kono, and Y. Matsuzawa. 1997. Requirement of Fas for the development of autoimmune diabetes in nonobese diabetic mice. *J Exp Med* 186:613-618.

Izui, S., V.E. Kelley, K. Masuda, H. Yoshida, J.B. Roths, and E.D. Murphy. 1984. Induction of various autoantibodies by mutant gene lpr in several strains of mice. *J Immunol* 133:227-233.

Jahng, A.W., I. Maricic, B. Pedersen, N. Burdin, O. Naidenko, M. Kronenberg, Y. Koezuka, and V. Kumar. 2001. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* 194:1789-1799.

Jansen, A., M. van Hagen, and H.A. Drexhage. 1995. Defective maturation and function of antigen-presenting cells in type 1 diabetes. *Lancet* 345:491-492.

Janssen, O., J. Qian, A. Linkermann, and D. Kabelitz. 2003. CD95 ligand--death factor and costimulatory molecule? *Cell death and differentiation* 10:1215-1225.

Jordan, M.A., J.M. Fletcher, D. Pellicci, and A.G. Baxter. 2007. Slamf1, the NKT cell control gene Nkt1. *J Immunol* 178:1618-1627.

Joseph, J., S. Bittner, F.M. Kaiser, H. Wiendl, and S. Kissler. 2012. IL-17 silencing does not protect nonobese diabetic mice from autoimmune diabetes. *J Immunol* 188:216-221.

Ju, S.T., D.J. Panka, H. Cui, R. Ettinger, M. el-Khatib, D.H. Sherr, B.Z. Stanger, and A. Marshak-Rothstein. 1995. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature* 373:444-448.

Jun, H.S., C.S. Yoon, L. Zbytnuik, N. van Rooijen, and J.W. Yoon. 1999. The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Exp Med* 189:347-358.

Kagi, D., B. Odermatt, P.S. Ohashi, R.M. Zinkernagel, and H. Hengartner. 1996. Development of insulitis without diabetes in transgenic mice lacking perforin-dependent cytotoxicity. *J Exp Med* 183:2143-2152.

Kagi, D., B. Odermatt, P. Seiler, R.M. Zinkernagel, T.W. Mak, and H. Hengartner. 1997. Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. *J Exp Med* 186:989-997.

Kaminuma, O., F. Kitamura, N. Kitamura, M. Miyagishi, K. Taira, K. Yamamoto, O. Miura, and S. Miyatake. 2004. GATA-3 suppresses IFN-gamma promoter activity independently of binding to cis-regulatory elements. *FEBS letters* 570:63-68.

Kanagawa, O., G. Xu, A. Tevaarwerk, and B.A. Vaupel. 2000. Protection of nonobese diabetic mice from diabetes by gene(s) closely linked to IFN-gamma receptor loci. *J Immunol* 164:3919-3923.

Kang, S., E.J. Park, Y. Joe, E. Seo, M.K. Park, S.Y. Seo, H.Y. Chung, Y.H. Yoo, D.K. Kim, and H.J. Lee. 2010. Systemic delivery of TNF-related apoptosis-inducing ligand (TRAIL) elevates levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) and prevents type 1 diabetes in nonobese diabetic mice. *Endocrinology* 151:5638-5646.

Karopoulos, C., M.J. Rowley, C.J. Handley, and R.A. Strugnell. 1995. Antibody reactivity to mycobacterial 65 kDa heat shock protein: relevance to autoimmunity. *Journal of autoimmunity* 8:235-248.

Karumuthil-Melethil, S., N. Perez, R. Li, and C. Vasu. 2008. Induction of innate immune response through TLR2 and dectin 1 prevents type 1 diabetes. *J Immunol* 181:8323-8334.

Kasibhatla, S., T. Brunner, L. Genestier, F. Echeverri, A. Mahboubi, and D.R. Green. 1998. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-kappa B and AP-1. *Molecular cell* 1:543-551.

Kavurma, M.M., F.S. Santiago, E. Bonfoco, and L.M. Khachigian. 2001. Sp1 phosphorylation regulates apoptosis via extracellular FasL-Fas engagement. *The Journal of biological chemistry* 276:4964-4971.

Kavurma, M.M., M. Schoppet, Y.V. Bobryshev, L.M. Khachigian, and M.R. Bennett. 2008. TRAIL stimulates proliferation of vascular smooth muscle cells via activation of NF-kappaB and induction of insulin-like growth factor-1 receptor. *The Journal of biological chemistry* 283:7754-7762.

Kayagaki, N., N. Yamaguchi, F. Nagao, S. Matsuo, H. Maeda, K. Okumura, and H. Yagita. 1997. Polymorphism of murine Fas ligand that affects the biological activity. *Proc Natl Acad Sci U S A* 94:3914-3919.

Kayagaki, N., N. Yamaguchi, M. Nakayama, H. Eto, K. Okumura, and H. Yagita. 1999. Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of type I IFNs. *J Exp Med* 189:1451-1460.

Kieffer, L.J., J.M. Greally, I. Landres, S. Nag, Y. Nakajima, T. Kohwi-Shigematsu, and P.B. Kavathas. 2002. Identification of a candidate regulatory region in the human CD8 gene complex by colocalization of DNase I hypersensitive sites and matrix attachment regions which bind SATB1 and GATA-3. *J Immunol* 168:3915-3922.

Kim, H.S., M.S. Han, K.W. Chung, S. Kim, E. Kim, M.J. Kim, E. Jang, H.A. Lee, J. Youn, S. Akira, and M.S. Lee. 2007. Toll-like receptor 2 senses beta-cell death and contributes to the initiation of autoimmune diabetes. *Immunity* 27:321-333.

Kim, S., K.A. Kim, D.Y. Hwang, T.H. Lee, N. Kayagaki, H. Yagita, and M.S. Lee. 2000. Inhibition of autoimmune diabetes by Fas ligand: the paradox is solved. *J Immunol* 164:2931-2936.

Kim, Y.H., S. Kim, K.A. Kim, H. Yagita, N. Kayagaki, K.W. Kim, and M.S. Lee. 1999. Apoptosis of pancreatic beta-cells detected in accelerated diabetes of NOD mice: no role of Fas-Fas ligand interaction in autoimmune diabetes. *Eur J Immunol* 29:455-465.

Kirvan, C.A., S.E. Swedo, J.S. Heuser, and M.W. Cunningham. 2003. Mimicry and autoantibody-mediated neuronal cell signaling in Sydenham chorea. *Nat Med* 9:914-920.

Kishimoto, H., and J. Sprent. 2001. A defect in central tolerance in NOD mice. *Nat Immunol* 2:1025-1031.

Kishimoto, H., C.D. Surh, and J. Sprent. 1998. A role for Fas in negative selection of thymocytes in vivo. *J Exp Med* 187:1427-1438.

Kivity, S., N. Agmon-Levin, M. Blank, and Y. Shoenfeld. 2009. Infections and autoimmunity--friends or foes? *Trends Immunol* 30:409-414.

Klas, C., K.M. Debatin, R.R. Jonker, and P.H. Krammer. 1993. Activation interferes with the APO-1 pathway in mature human T cells. *International immunology* 5:625-630.

Klefstrom, J., E.W. Verschuren, and G. Evan. 2002. c-Myc augments the apoptotic activity of cytosolic death receptor signaling proteins by engaging the

mitochondrial apoptotic pathway. *The Journal of biological chemistry* 277:43224-43232.

Knight, J.G., and D.D. Adams. 1981. Genes determining autoimmune disease in New Zealand mice. *Journal of clinical & laboratory immunology* 5:165-170.

Ko, L.J., M. Yamamoto, M.W. Leonard, K.M. George, P. Ting, and J.D. Engel. 1991. Murine and human T-lymphocyte GATA-3 factors mediate transcription through a cis-regulatory element within the human T-cell receptor delta gene enhancer. *Molecular and cellular biology* 11:2778-2784.

Kobayashi, S., T. Hirano, M. Kakinuma, and T. Uede. 1993. Transcriptional repression and differential splicing of Fas mRNA by early transposon (ETn) insertion in autoimmune lpr mice. *Biochemical and biophysical research communications* 191:617-624.

Kodama, S., M. Davis, and D.L. Faustman. 2005. The therapeutic potential of tumor necrosis factor for autoimmune disease: a mechanistically based hypothesis. *Cellular and molecular life sciences : CMLS* 62:1850-1862.

Kodama, S., W. Kuhtreiber, S. Fujimura, E.A. Dale, and D.L. Faustman. 2003. Islet regeneration during the reversal of autoimmune diabetes in NOD mice. *Science* 302:1223-1227.

Komiyama, Y., S. Nakae, T. Matsuki, A. Nambu, H. Ishigame, S. Kakuta, K. Sudo, and Y. Iwakura. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 177:566-573.

Kommajosyula, S., S. Reddy, K. Nitschke, J.R. Kanwar, M. Karanam, and G.W. Krissansen. 2001. Leukocytes infiltrating the pancreatic islets of nonobese diabetic mice are transformed into inactive exiles by combinational anti-cell adhesion therapy. *Journal of leukocyte biology* 70:510-517.

Krammer, P.H. 2000. CD95's deadly mission in the immune system. *Nature* 407:789-795.

Kreuwel, H.T., D.J. Morgan, T. Krahl, A. Ko, N. Sarvetnick, and L.A. Sherman. 1999. Comparing the relative role of perforin/granzyme versus Fas/Fas ligand cytotoxic pathways in CD8+ T cell-mediated insulin-dependent diabetes mellitus. *J Immunol* 163:4335-4341.

Kuriya, G., T. Uchida, S. Akazawa, M. Kobayashi, K. Nakamura, T. Satoh, I. Horie, E. Kawasaki, H. Yamasaki, L. Yu, Y. Iwakura, H. Sasaki, Y. Nagayama, A.

Kawakami, and N. Abiru. 2013. Double deficiency in IL-17 and IFN-gamma signalling significantly suppresses the development of diabetes in the NOD mouse. *Diabetologia* 56:1773-1780.

Kurrer, M.O., S.V. Pakala, H.L. Hanson, and J.D. Katz. 1997. Beta cell apoptosis in T cell-mediated autoimmune diabetes. *Proc Natl Acad Sci U S A* 94:213-218.

Laitinen, M.P., M. Anttonen, I. Ketola, D.B. Wilson, O. Ritvos, R. Butzow, and M. Heikinheimo. 2000. Transcription factors GATA-4 and GATA-6 and a GATA family cofactor, FOG-2, are expressed in human ovary and sex cord-derived ovarian tumors. *The Journal of clinical endocrinology and metabolism* 85:3476-3483.

Lamhamedi-Cherradi, S.E., S. Zheng, R.M. Tisch, and Y.H. Chen. 2003a. Critical roles of tumor necrosis factor-related apoptosis-inducing ligand in type 1 diabetes. *Diabetes* 52:2274-2278.

Lamhamedi-Cherradi, S.E., S.J. Zheng, K.A. Maguschak, J. Peschon, and Y.H. Chen. 2003b. Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL-/- mice. *Nat Immunol* 4:255-260.

Latinis, K.M., L.A. Norian, S.L. Eliason, and G.A. Koretzky. 1997. Two NFAT transcription factor binding sites participate in the regulation of CD95 (Fas) ligand expression in activated human T cells. *The Journal of biological chemistry* 272:31427-31434.

Lee, G.R., P.E. Fields, and R.A. Flavell. 2001. Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level. *Immunity* 14:447-459.

Lee, I.F., H. Qin, J.J. Priatel, and R. Tan. 2008. Critical role for IFN-gamma in natural killer cell-mediated protection from diabetes. *Eur J Immunol* 38:82-89.

Lee, I.F., H. Qin, J. Trudeau, J. Dutz, and R. Tan. 2004. Regulation of autoimmune diabetes by complete Freund's adjuvant is mediated by NK cells. *J Immunol* 172:937-942.

Lee, I.F., P. van den Elzen, R. Tan, and J.J. Priatel. 2011. NKT cells are required for complete Freund's adjuvant-mediated protection from autoimmune diabetes. *J Immunol* 187:2898-2904.

Leite-de-Moraes, M.C., A. Herbelin, C. Gouarin, Y. Koezuka, E. Schneider, and M. Dy. 2000. Fas/Fas ligand interactions promote activation-induced cell death of NK T lymphocytes. *J Immunol* 165:4367-4371.

Lenzi, M., G. Ballardini, M. Fusconi, F. Cassani, L. Selleri, U. Volta, D. Zauli, and F.B. Bianchi. 1990. Type 2 autoimmune hepatitis and hepatitis C virus infection. *Lancet* 335:258-259.

Lesage, S., S.B. Hartley, S. Akkaraju, J. Wilson, M. Townsend, and C.C. Goodnow. 2002. Failure to censor forbidden clones of CD4 T cells in autoimmune diabetes. *J Exp Med* 196:1175-1188.

Levinson, G., and G.A. Gutman. 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Molecular biology and evolution* 4:203-221.

Lien, E., T.J. Sellati, A. Yoshimura, T.H. Flo, G. Rawadi, R.W. Finberg, J.D. Carroll, T. Espevik, R.R. Ingalls, J.D. Radolf, and D.T. Golenbock. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *The Journal of biological chemistry* 274:33419-33425.

Liston, A., S. Lesage, J. Wilson, L. Peltonen, and C.C. Goodnow. 2003. Aire regulates negative selection of organ-specific T cells. *Nat Immunol* 4:350-354.

Lit, L.C., C.K. Wong, L.S. Tam, E.K. Li, and C.W. Lam. 2006. Raised plasma concentration and ex vivo production of inflammatory chemokines in patients with systemic lupus erythematosus. *Annals of the rheumatic diseases* 65:209-215.

Litherland, S.A., X.T. Xie, A.D. Hutson, C. Wasserfall, D.S. Whittaker, J.X. She, A. Hofig, M.A. Dennis, K. Fuller, R. Cook, D. Schatz, L.L. Moldawer, and M.J. Clare-Salzler. 1999. Aberrant prostaglandin synthase 2 expression defines an antigen-presenting cell defect for insulin-dependent diabetes mellitus. *J Clin Invest* 104:515-523.

Littorin, B., P. Blom, A. Scholin, H.J. Arnqvist, G. Blohme, J. Bolinder, A. Ekbom-Schnell, J.W. Eriksson, S. Gudbjornsdottir, L. Nystrom, J. Ostman, and G. Sundkvist. 2006. Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS). *Diabetologia* 49:2847-2852.

Liu, S., H. Wang, Y. Jin, R. Podolsky, M.V. Reddy, J. Pedersen, B. Bode, J. Reed, D. Steed, S. Anderson, P. Yang, A. Muir, L. Steed, D. Hopkins, Y. Huang, S. Purohit, C.Y. Wang, A.K. Steck, A. Montemari, G. Eisenbarth, M. Rewers, and J.X. She. 2009. IFIH1 polymorphisms are significantly associated with type 1 diabetes and IFIH1 gene expression in peripheral blood mononuclear cells. *Human molecular genetics* 18:358-365.

Loetscher, M., B. Gerber, P. Loetscher, S.A. Jones, L. Piali, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1996. Chemokine receptor specific for IP10 and mig: structure, function, and expression in activated T-lymphocytes. *J Exp Med* 184:963-969.

Lohmann, T., S. Laue, U. Nietzschmann, T.M. Kapellen, I. Lehmann, S. Schroeder, R. Paschke, and W. Kiess. 2002. Reduced expression of Th1-associated chemokine receptors on peripheral blood lymphocytes at diagnosis of type 1 diabetes. *Diabetes* 51:2474-2480.

Loutit, J.F., and P.L. Mollison. 1946. Haemolytic icterus (acholuric jaundice) congenital and acquired. *The Journal of pathology and bacteriology* 58:711-728.

Loweth, A.C., G.T. Williams, R.F. James, J.H. Scarpello, and N.G. Morgan. 1998. Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1beta and Fas ligation. *Diabetes* 47:727-732.

Luckerath, K., V. Kirkin, I.M. Melzer, F.B. Thalheimer, D. Siele, W. Milani, T. Adler, A. Aguilar-Pimentel, M. Horsch, G. Michel, J. Beckers, D.H. Busch, M. Ollert, V. Gailus-Durner, H. Fuchs, M. Hrabe de Angelis, F.J. Staal, K. Rajalingam, A.O. Hueber, L.J. Strobl, U. Zimber-Strobl, and M. Zornig. 2011. Immune modulation by Fas ligand reverse signaling: lymphocyte proliferation is attenuated by the intracellular Fas ligand domain. *Blood* 117:519-529.

Luo, Y., X. Chen, and M.A. O'Donnell. 2007. Mycobacterium bovis bacillus Calmette-Guerin (BCG) induces human CC- and CXC-chemokines in vitro and in vivo. *Clinical and experimental immunology* 147:370-378.

Luster, A.D., and J.V. Ravetch. 1987. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med* 166:1084-1097.

Lyon, M.F., J. Peters, P.H. Glenister, S. Ball, and E. Wright. 1990. The scurfy mouse mutant has previously unrecognized hematological abnormalities and

resembles Wiskott-Aldrich syndrome. *Proc Natl Acad Sci U S A* 87:2433-2437.

MacDonald, G.C., V.N. Kakkanaiah, E.S. Sobel, P.L. Cohen, and R.A. Eisenberg. 1995. In vivo depletion of Thy-1-positive cells originating from normal bone marrow abrogates the suppression of gld disease in normal-gld mixed bone marrow chimeras. *J Immunol* 154:444-449.

MacFarlane, M., M. Ahmad, S.M. Srinivasula, T. Fernandes-Alnemri, G.M. Cohen, and E.S. Alnemri. 1997. Identification and molecular cloning of two novel receptors for the cytotoxic ligand TRAIL. *The Journal of biological chemistry* 272:25417-25420.

Mack, P., and J. Freedman. 2000. Autoimmune hemolytic anemia: a history. *Transfusion medicine reviews* 14:223-233.

Mackay, I.R. 2009. Clustering and commonalities among autoimmune diseases. *Journal of autoimmunity* 33:170-177.

MacKay, P., J. Jacobson, and A. Rabinovitch. 1986. Spontaneous diabetes mellitus in the Bio-Breeding/Worcester rat. Evidence in vitro for natural killer cell lysis of islet cells. *J Clin Invest* 77:916-924.

Manirarora, J.N., M.M. Kosiewicz, S.A. Parnell, and P. Alard. 2008. APC activation restores functional CD4(+)CD25(+) regulatory T cells in NOD mice that can prevent diabetes development. *PloS one* 3:e3739.

Marine, J.C., D.J. Topham, C. McKay, D. Wang, E. Parganas, D. Stravopodis, A. Yoshimura, and J.N. Ihle. 1999. SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* 98:609-616.

Markine-Goriaynoff, D., J.T. van der Logt, C. Truyens, T.D. Nguyen, F.W. Heessen, G. Bigaignon, Y. Carlier, and J.P. Coutelier. 2000. IFN-gamma-independent IgG2a production in mice infected with viruses and parasites. *International immunology* 12:223-230.

Martin-Orozco, N., Y. Chung, S.H. Chang, Y.H. Wang, and C. Dong. 2009. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. *Eur J Immunol* 39:216-224.

Martinez-Lorenzo, M.J., M.A. Alava, S. Gamen, K.J. Kim, A. Chuntharapai, A. Pineiro, J. Naval, and A. Anel. 1998. Involvement of APO2 ligand/TRAIL in

activation-induced death of Jurkat and human peripheral blood T cells. *Eur J Immunol* 28:2714-2725.

Martins, T.C., and A.P. Aguas. 1999. Mechanisms of Mycobacterium avium-induced resistance against insulin-dependent diabetes mellitus (IDDM) in non-obese diabetic (NOD) mice: role of Fas and Th1 cells. *Clinical and experimental immunology* 115:248-254.

Maruyama, T., K. Watanabe, I. Takei, A. Kasuga, A. Shimada, T. Yanagawa, T. Kasatani, Y. Suzuki, K. Kataoka, Saruta, and et al. 1991. Anti-asialo GM1 antibody suppression of cyclophosphamide-induced diabetes in NOD mice. *Diabetes research* 17:37-41.

Masuda, A., Y. Yoshikai, H. Kume, and T. Matsuguchi. 2004. The interaction between GATA proteins and activator protein-1 promotes the transcription of IL-13 in mast cells. *J Immunol* 173:5564-5573.

Mathis, D., L. Vence, and C. Benoist. 2001. beta-Cell death during progression to diabetes. *Nature* 414:792-798.

Matsuda, J.L., O.V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C.R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* 192:741-754.

Matsui, K., A. Fine, B. Zhu, A. Marshak-Rothstein, and S.T. Ju. 1998. Identification of two NF-kappa B sites in mouse CD95 ligand (Fas ligand) promoter: functional analysis in T cell hybridoma. *J Immunol* 161:3469-3473.

Mazza, G., M.E. el Idrissi, J.P. Coutelier, A. Corato, C.J. Elson, C.J. Pfau, and M.J. Day. 1997. Infection of C3HeB/FeJ mice with the docile strain of lymphocytic choriomeningitis virus induces autoantibodies specific for erythrocyte Band 3. *Immunology* 91:239-245.

McKinney, P.A., R. Parslow, K.A. Gurney, G.R. Law, H.J. Bodansky, and R. Williams. 1999. Perinatal and neonatal determinants of childhood type 1 diabetes. A case-control study in Yorkshire, U.K. *Diabetes care* 22:928-932.

McMurray, R.W., and K. Elbourne. 1997. Hepatitis C virus infection and autoimmunity. *Semin Arthritis Rheum* 26:689-701.

McNeil, M.R., and P.J. Brennan. 1991. Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis

and drug resistance; some thoughts and possibilities arising from recent structural information. *Research in microbiology* 142:451-463.

Mi, Q.S., D. Ly, S.E. Lamhamedi-Cherradi, K.V. Salojin, L. Zhou, M. Grattan, C. Meagher, P. Zucker, Y.H. Chen, J. Nagle, D. Taub, and T.L. Delovitch. 2003. Blockade of tumor necrosis factor-related apoptosis-inducing ligand exacerbates type 1 diabetes in NOD mice. *Diabetes* 52:1967-1975.

Michels, A.W., and M. von Herrath. 2011. 2011 Update: antigen-specific therapy in type 1 diabetes. *Current opinion in endocrinology, diabetes, and obesity* 18:235-240.

Migliaccio, A.R., Y. Jiang, G. Migliaccio, S. Nicolis, S. Crotta, A. Ronchi, S. Ottolenghi, and J.W. Adamson. 1993. Transcriptional and posttranscriptional regulation of the expression of the erythropoietin receptor gene in human erythropoietin-responsive cell lines. *Blood* 82:3760-3769.

Minnikin, D.E. 1991. Chemical principles in the organization of lipid components in the mycobacterial cell envelope. *Research in microbiology* 142:423-427.

Mittrucker, H.W., T. Matsuyama, A. Grossman, T.M. Kundig, J. Potter, A. Shahinian, A. Wakeham, B. Patterson, P.S. Ohashi, and T.W. Mak. 1997. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science* 275:540-543.

Miwa, K., M. Asano, R. Horai, Y. Iwakura, S. Nagata, and T. Suda. 1998. Caspase 1-independent IL-1beta release and inflammation induced by the apoptosis inducer Fas ligand. *Nat Med* 4:1287-1292.

Mohamood, A.S., M.L. Guler, Z. Xiao, D. Zheng, A. Hess, Y. Wang, H. Yagita, J.P. Schneck, and A.R. Hamad. 2007. Protection from autoimmune diabetes and T-cell lymphoproliferation induced by FasL mutation are differentially regulated and can be uncoupled pharmacologically. *The American journal of pathology* 171:97-106.

Molkentin, J.D. 2000. The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. *The Journal of biological chemistry* 275:38949-38952.

Moore, F., N. Naamane, M.L. Colli, T. Bouckenooghe, F. Ortis, E.N. Gurzov, M. Igoillo-Esteve, C. Mathieu, G. Bontempi, T. Thykjaer, T.F. Orntoft, and D.L. Eizirik.

2011. STAT1 is a master regulator of pancreatic {beta}-cell apoptosis and islet inflammation. *The Journal of biological chemistry* 286:929-941.

Mordes, J., D. Serreze, D. Greiner, and A. Rossini. 2004. Animal models of autoimmune diabetes mellitus. In: Diabetes Mellitus: A Fundamental and Clinical Text. 3rd Edition, LeRoith D (ed). :591-610.

Mori, Y., T. Kodaka, T. Kato, E.M. Kanagawa, and O. Kanagawa. 2009. Critical role of IFN-gamma in CFA-mediated protection of NOD mice from diabetes development. *International immunology* 21:1291-1299.

Morimoto, J., H. Yoneyama, A. Shimada, T. Shigihara, S. Yamada, Y. Oikawa, K. Matsushima, T. Saruta, and S. Narumi. 2004. CXC chemokine ligand 10 neutralization suppresses the occurrence of diabetes in nonobese diabetic mice through enhanced beta cell proliferation without affecting insulitis. *J Immunol* 173:7017-7024.

Moriwaki, M., N. Itoh, J. Miyagawa, K. Yamamoto, A. Imagawa, K. Yamagata, H. Iwahashi, H. Nakajima, M. Namba, S. Nagata, T. Hanafusa, and Y. Matsuzawa. 1999. Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset Type I diabetes mellitus. *Diabetologia* 42:1332-1340.

Murakami, M., K. Nakajima, K. Yamazaki, T. Muraguchi, T. Serikawa, and T. Honjo. 1997. Effects of breeding environments on generation and activation of autoreactive B-1 cells in anti-red blood cell autoantibody transgenic mice. *J Exp Med* 185:791-794.

Murakami, M., T. Tsubata, M. Okamoto, A. Shimizu, S. Kumagai, H. Imura, and T. Honjo. 1992. Antigen-induced apoptotic death of Ly-1 B cells responsible for autoimmune disease in transgenic mice. *Nature* 357:77-80.

Murakami, M., T. Tsubata, R. Shinkura, S. Nisitani, M. Okamoto, H. Yoshioka, T. Usui, S. Miyawaki, and T. Honjo. 1994. Oral administration of lipopolysaccharides activates B-1 cells in the peritoneal cavity and lamina propria of the gut and induces autoimmune symptoms in an autoantibody transgenic mouse. *J Exp Med* 180:111-121.

Musaji, A., F. Cormont, G. Thirion, C.L. Cambiaso, and J.P. Coutelier. 2004. Exacerbation of autoantibody-mediated thrombocytopenic purpura by infection with mouse viruses. *Blood* 104:2102-2106.

Nagamine, K., P. Peterson, H.S. Scott, J. Kudoh, S. Minoshima, M. Heino, K.J. Krohn, M.D. Lalioti, P.E. Mullis, S.E. Antonarakis, K. Kawasaki, S. Asakawa, F. Ito, and N. Shimizu. 1997. Positional cloning of the APECED gene. *Nat Genet* 17:393-398.

Nagata, S., and P. Golstein. 1995. The Fas death factor. *Science* 267:1449-1456.

Nagata, S., and T. Suda. 1995. Fas and Fas ligand: lpr and gld mutations. *Immunol Today* 16:39-43.

Nakayama, M., M. Nagata, H. Yasuda, K. Arisawa, R. Kotani, K. Yamada, S.A. Chowdhury, S. Chakrabarty, Z.Z. Jin, H. Yagita, K. Yokono, and M. Kasuga. 2002. Fas/Fas ligand interactions play an essential role in the initiation of murine autoimmune diabetes. *Diabetes* 51:1391-1397.

Narumi, S., T. Takeuchi, Y. Kobayashi, and K. Konishi. 2000. Serum levels of ifn-inducible PROTEIN-10 relating to the activity of systemic lupus erythematosus. *Cytokine* 12:1561-1565.

Naysmith, J.D., C.J. Elson, M. Dallman, E. Fletcher, and M.G. Ortega-Pierres. 1980. Anti-erythrocyte autoantibody production in mice associated with the injection of rat erythrocytes. *Immunology* 39:469-479.

Nejentsev, S., N. Walker, D. Riches, M. Egholm, and J.A. Todd. 2009. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science* 324:387-389.

Nesterov, A., M. Nikrad, T. Johnson, and A.S. Kraft. 2004. Oncogenic Ras sensitizes normal human cells to tumor necrosis factor-alpha-related apoptosis-inducing ligand-induced apoptosis. *Cancer research* 64:3922-3927.

Newton, K., A.W. Harris, M.L. Bath, K.G. Smith, and A. Strasser. 1998. A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *The EMBO journal* 17:706-718.

Nicoletti, F., I. Conget, M. Di Mauro, R. Di Marco, M.C. Mazzarino, K. Bendtzen, A. Messina, and R. Gomis. 2002. Serum concentrations of the interferon-gamma-inducible chemokine IP-10/CXCL10 are augmented in both newly diagnosed Type I diabetes mellitus patients and subjects at risk of developing the disease. *Diabetologia* 45:1107-1110.

Nicoletti, F., P. Zaccone, R. Di Marco, M. Di Mauro, G. Magro, S. Grasso, L. Mughini, P. Meroni, and G. Garotta. 1996. The effects of a nonimmunogenic form of murine soluble interferon-gamma receptor on the development of autoimmune diabetes in the NOD mouse. *Endocrinology* 137:5567-5575.

Nieda, M., A. Nicol, Y. Koezuka, A. Kikuchi, N. Lapteva, Y. Tanaka, K. Tokunaga, K. Suzuki, N. Kayagaki, H. Yagita, H. Hirai, and T. Juji. 2001. TRAIL expression by activated human CD4(+)V alpha 24NKT cells induces in vitro and in vivo apoptosis of human acute myeloid leukemia cells. *Blood* 97:2067-2074.

Nielsen, N., N. Odum, B. Urso, L.L. Lanier, and P. Spee. 2012. Cytotoxicity of CD56(bright) NK cells towards autologous activated CD4+ T cells is mediated through NKG2D, LFA-1 and TRAIL and dampened via CD94/NKG2A. *PloS one* 7:e31959.

Nikoopour, E., J.A. Schwartz, K. Huszarik, C. Sandrock, O. Krougly, E. Lee-Chan, and B. Singh. 2010. Th17 polarized cells from nonobese diabetic mice following mycobacterial adjuvant immunotherapy delay type 1 diabetes. *J Immunol* 184:4779-4788.

Nikoopour, E., J.A. Schwartz, and B. Singh. 2008. Therapeutic benefits of regulating inflammation in autoimmunity. *Inflammation & allergy drug targets* 7:203-210.

Nishio, J., J.L. Gaglia, S.E. Turvey, C. Campbell, C. Benoist, and D. Mathis. 2006. Islet recovery and reversal of murine type 1 diabetes in the absence of any infused spleen cell contribution. *Science* 311:1775-1778.

Novelli, F., P. Bernabei, L. Ozmen, L. Rigamonti, A. Allione, S. Pestka, G. Garotta, and G. Forni. 1996. Switching on of the proliferation or apoptosis of activated human T lymphocytes by IFN-gamma is correlated with the differential expression of the alpha- and beta-chains of its receptor. *J Immunol* 157:1935-1943.

Nowak, M., F. Kopp, K. Roelofs-Haarhuis, X. Wu, and E. Gleichmann. 2006. Oral nickel tolerance: Fas ligand-expressing invariant NK T cells promote tolerance induction by eliciting apoptotic death of antigen-carrying, effete B cells. *J Immunol* 176:4581-4589.

O'Connor, W., Jr., M. Kamanaka, C.J. Booth, T. Town, S. Nakae, Y. Iwakura, J.K. Kolls, and R.A. Flavell. 2009. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat Immunol* 10:603-609.

Ochiai, K., S. Ozaki, A. Tanino, S. Watanabe, T. Ueno, K. Mitsui, J. Toei, Y. Inada, S. Hirose, T. Shirai, and H. Nishimura. 2000. Genetic regulation of anti-erythrocyte autoantibodies and splenomegaly in autoimmune hemolytic anemia-prone new zealand black mice. *International immunology* 12:1-8.

Ogasawara, K., J.A. Hamerman, L.R. Ehrlich, H. Bour-Jordan, P. Santamaria, J.A. Bluestone, and L.L. Lanier. 2004. NKG2D blockade prevents autoimmune diabetes in NOD mice. *Immunity* 20:757-767.

Okamoto, M., M. Murakami, A. Shimizu, S. Ozaki, T. Tsubata, S. Kumagai, and T. Honjo. 1992. A transgenic model of autoimmune hemolytic anemia. *J Exp Med* 175:71-79.

Oldstone, M.B. 1988. Prevention of type I diabetes in nonobese diabetic mice by virus infection. *Science* 239:500-502.

Onkamo, P., S. Vaananen, M. Karvonen, and J. Tuomilehto. 1999. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. *Diabetologia* 42:1395-1403.

Orkin, S.H. 1992. GATA-binding transcription factors in hematopoietic cells. *Blood* 80:575-581.

Ou, D., D.L. Metzger, X. Wang, J. Huang, P. Pozzilli, and A.J. Tingle. 2002. TNF-related apoptosis-inducing ligand death pathway-mediated human beta-cell destruction. *Diabetologia* 45:1678-1688.

Ou, D., L.A. Mitchell, D.L. Metzger, S. Gillam, and A.J. Tingle. 2000. Cross-reactive rubella virus and glutamic acid decarboxylase (65 and 67) protein determinants recognised by T cells of patients with type I diabetes mellitus. *Diabetologia* 43:750-762.

Oura, R., R. Arakaki, A. Yamada, Y. Kudo, E. Tanaka, Y. Hayashi, and N. Ishimaru. 2013. Induction of rapid T cell death and phagocytic activity by Fas-deficient lpr macrophages. *J Immunol* 190:578-585.

Owen-Schaub, L.B., S. Yonehara, W.L. Crump, 3rd, and E.A. Grimm. 1992. DNA fragmentation and cell death is selectively triggered in activated human

lymphocytes by Fas antigen engagement. *Cellular immunology* 140:197-205.

Pai, R.K., M.E. Pennini, A.A. Tobian, D.H. Canaday, W.H. Boom, and C.V. Harding. 2004. Prolonged toll-like receptor signaling by Mycobacterium tuberculosis and its 19-kilodalton lipoprotein inhibits gamma interferon-induced regulation of selected genes in macrophages. *Infection and immunity* 72:6603-6614.

Pan, G., K. O'Rourke, A.M. Chinnaiyan, R. Gentz, R. Ebner, J. Ni, and V.M. Dixit. 1997. The receptor for the cytotoxic ligand TRAIL. *Science* 276:111-113.

Patterson, C.C., G. Dahlquist, V. Harjutsalo, G. Joner, R.G. Feltbower, J. Svensson, E. Schober, E. Gyurus, C. Castell, B. Urbonaite, J. Rosenbauer, V. Iotova, A.V. Thorsson, and G. Soltesz. 2007. Early mortality in EURODIAB population-based cohorts of type 1 diabetes diagnosed in childhood since 1989. *Diabetologia* 50:2439-2442.

Patterson, C.C., G. Dahlquist, G. Soltesz, and A. Green. 2001. Is childhood-onset type I diabetes a wealth-related disease? An ecological analysis of European incidence rates. *Diabetologia* 44 Suppl 3:B9-16.

Patterson, C.C., G.G. Dahlquist, E. Gyurus, A. Green, and G. Soltesz. 2009. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. *Lancet* 373:2027-2033.

Peakman, M., and M. von Herrath. 2010. Antigen-specific immunotherapy for type 1 diabetes: maximizing the potential. *Diabetes* 59:2087-2093.

Pender, M.P. 2003. Infection of autoreactive B lymphocytes with EBV, causing chronic autoimmune diseases. *Trends Immunol* 24:584-588.

Peng, R.H., E. Paek, C.Q. Xia, N. Tennyson, and M.J. Clare-Salzler. 2006. Heightened interferon-alpha/beta response causes myeloid cell dysfunction and promotes T1D pathogenesis in NOD mice. *Annals of the New York Academy of Sciences* 1079:99-102.

Pennini, M.E., R.K. Pai, D.C. Schultz, W.H. Boom, and C.V. Harding. 2006. Mycobacterium tuberculosis 19-kDa lipoprotein inhibits IFN-gamma-induced chromatin remodeling of MHC2TA by TLR2 and MAPK signaling. *J Immunol* 176:4323-4330.

Peter, M.E., P. Legembre, and B.C. Barnhart. 2005. Does CD95 have tumor promoting activities? *Biochimica et biophysica acta* 1755:25-36.

Petrovsky, N., D. Silva, L. Socha, R. Slattery, and B. Charlton. 2002. The role of Fas ligand in beta cell destruction in autoimmune diabetes of NOD mice. *Annals of the New York Academy of Sciences* 958:204-208.

Piccirillo, C.A., M. Tritt, E. Sgouroudis, A. Albanese, M. Pyzik, and V. Hay. 2005. Control of type 1 autoimmune diabetes by naturally occurring CD4+CD25+ regulatory T lymphocytes in neonatal NOD mice. *Annals of the New York Academy of Sciences* 1051:72-87.

Pirofsky, B. 1976. Clinical aspects of autoimmune hemolytic anemia. *Seminars in hematology* 13:251-265.

Pitti, R.M., S.A. Marsters, S. Ruppert, C.J. Donahue, A. Moore, and A. Ashkenazi. 1996. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *The Journal of biological chemistry* 271:12687-12690.

Playfair, J.H., and S. Marshall-Clarke. 1973. Induction of red cell autoantibodies in normal mice. *Nature: New biology* 243:213-214.

Podolin, P.L., P. Denny, C.J. Lord, N.J. Hill, J.A. Todd, L.B. Peterson, L.S. Wicker, and P.A. Lyons. 1997. Congenic mapping of the insulin-dependent diabetes (Idd) gene, Idd10, localizes two genes mediating the Idd10 effect and eliminates the candidate Fcgr1. *J Immunol* 159:1835-1843.

Poirot, L., C. Benoist, and D. Mathis. 2004. Natural killer cells distinguish innocuous and destructive forms of pancreatic islet autoimmunity. *Proc Natl Acad Sci U S A* 101:8102-8107.

Pozzilli, P. 1997. BCG vaccine in insulin-dependent diabetes mellitus. IMDIAB Group. *Lancet* 349:1520-1521.

Prud'homme, G.J., D.H. Kono, and A.N. Theofilopoulos. 1995. Quantitative polymerase chain reaction analysis reveals marked overexpression of interleukin-1 beta, interleukin-1 and interferon-gamma mRNA in the lymph nodes of lupus-prone mice. *Molecular immunology* 32:495-503.

Qin, H.Y., P. Chaturvedi, and B. Singh. 2004. In vivo apoptosis of diabetogenic T cells in NOD mice by IFN-gamma/TNF-alpha. *International immunology* 16:1723-1732.

Qin, H.Y., M.W. Sadelain, C. Hitchon, J. Lauzon, and B. Singh. 1993. Complete Freund's adjuvant-induced T cells prevent the development and adoptive transfer of diabetes in nonobese diabetic mice. *J Immunol* 150:2072-2080.

Quintana, F.J., A. Rotem, P. Carmi, and I.R. Cohen. 2000. Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. *J Immunol* 165:6148-6155.

Rainbow, D.B., L. Esposito, S.K. Howlett, K.M. Hunter, J.A. Todd, L.B. Peterson, and L.S. Wicker. 2008. Commonality in the genetic control of Type 1 diabetes in humans and NOD mice: variants of genes in the IL-2 pathway are associated with autoimmune diabetes in both species. *Biochem Soc Trans* 36:312-315.

Ramchandran, R., C. Bengra, B. Whitney, K. Lanclos, and D. Tuan. 2000. A (GATA)(7) motif located in the 5' boundary area of the human beta-globin locus control region exhibits silencer activity in erythroid cells. *American journal of hematology* 65:14-24.

Rastogi, N. 1991. Recent observations concerning structure and function relationships in the mycobacterial cell envelope: elaboration of a model in terms of mycobacterial pathogenicity, virulence and drug-resistance. *Research in microbiology* 142:464-476.

Rathmell, J.C., M.P. Cooke, W.Y. Ho, J. Grein, S.E. Townsend, M.M. Davis, and C.C. Goodnow. 1995. CD95 (Fas)-dependent elimination of self-reactive B cells upon interaction with CD4+ T cells. *Nature* 376:181-184.

Redd, S., S. Ginn, and J.M. Ross. 2002. Fas and Fas ligand immunolocalization in pancreatic islets of NOD mice during spontaneous and cyclophosphamide-accelerated diabetes. *The Histochemical journal* 34:1-12.

Redondo, M.J., M. Rewers, L. Yu, S. Garg, C.C. Pilcher, R.B. Elliott, and G.S. Eisenbarth. 1999. Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-twin siblings of patients with type 1 diabetes: prospective twin study. *Bmj* 318:698-702.

Ren, X., F. Ye, Z. Jiang, Y. Chu, S. Xiong, and Y. Wang. 2007. Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. *Cell death and differentiation* 14:2076-2084.

Rewers, M. 1991. The changing face of the epidemiology of insulin-dependent diabetes mellitus (IDDM): research designs and models of disease causation. *Ann Med* 23:419-426.

Rhoades, E., F. Hsu, J.B. Torrelles, J. Turk, D. Chatterjee, and D.G. Russell. 2003. Identification and macrophage-activating activity of glycolipids released from intracellular Mycobacterium bovis BCG. *Molecular microbiology* 48:875-888.

Rhode, A., M.E. Pauza, A.M. Barral, E. Rodrigo, M.B. Oldstone, M.G. von Herrath, and U. Christen. 2005. Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. *J Immunol* 175:3516-3524.

Rieger, R., and M.E. Gershwin. 2007. The X and why of xenobiotics in primary biliary cirrhosis. *Journal of autoimmunity* 28:76-84.

Rieux-Laucat, F., F. Le Deist, C. Hivroz, I.A. Roberts, K.M. Debatin, A. Fischer, and J.P. de Villartay. 1995. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* 268:1347-1349.

Roberts, A.I., S. Devadas, X. Zhang, L. Zhang, A. Keegan, K. Greeneltch, J. Solomon, L. Wei, J. Das, E. Sun, C. Liu, Z. Yuan, J.N. Zhou, and Y. Shi. 2003. The role of activation-induced cell death in the differentiation of T-helper-cell subsets. *Immunologic research* 28:285-293.

Rodacki, M., B. Svoren, V. Butty, W. Besse, L. Laffel, C. Benoist, and D. Mathis. 2007. Altered natural killer cells in type 1 diabetic patients. *Diabetes* 56:177-185.

Rodrigues, A., R.D. Coelho, and F. Pereira. 1990. [Lupus vulgaris after BCG vaccination. A clinical case]. *Med Cutan Ibero Lat Am* 18:224-226.

Roep, B.O., F.S. Kleijwegt, A.G. van Halteren, V. Bonato, U. Boggi, F. Vendrame, P. Marchetti, and F. Dotta. 2010. Islet inflammation and CXCL10 in recent-onset type 1 diabetes. *Clinical and experimental immunology* 159:338-343.

Rossini, A.A., R.M. Williams, J.P. Mordes, M.C. Appel, and A.A. Like. 1979. Spontaneous diabetes in the gnotobiotic BB/W rat. *Diabetes* 28:1031-1032.

Roths, J.B., E.D. Murphy, and E.M. Eicher. 1984. A new mutation, gld, that produces lymphoproliferation and autoimmunity in C3H/HeJ mice. *J Exp Med* 159:1-20.

Rothstein, T.L., J.K. Wang, D.J. Panka, L.C. Foote, Z. Wang, B. Stanger, H. Cui, S.T. Ju, and A. Marshak-Rothstein. 1995. Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. *Nature* 374:163-165.

Rulong, S., A.P. Aguas, P.P. da Silva, and M.T. Silva. 1991. Intramacrophagic Mycobacterium avium bacilli are coated by a multiple lamellar structure: freeze fracture analysis of infected mouse liver. *Infection and immunity* 59:3895-3902.

Rus, V., V. Nguyen, R. Puliaev, I. Puliaeva, V. Zernetkina, I. Luzina, J.C. Papadimitriou, and C.S. Via. 2007. T cell TRAIL promotes murine lupus by sustaining effector CD4 Th cell numbers and by inhibiting CD8 CTL activity. *J Immunol* 178:3962-3972.

Russell, J.H., B. Rush, C. Weaver, and R. Wang. 1993. Mature T cells of autoimmune lpr/lpr mice have a defect in antigen-stimulated suicide. *Proc Natl Acad Sci U S A* 90:4409-4413.

Russell, J.H., and R. Wang. 1993. Autoimmune gld mutation uncouples suicide and cytokine/proliferation pathways in activated, mature T cells. *Eur J Immunol* 23:2379-2382.

Ryu, S., S. Kodama, K. Ryu, D.A. Schoenfeld, and D.L. Faustman. 2001. Reversal of established autoimmune diabetes by restoration of endogenous beta cell function. *J Clin Invest* 108:63-72.

Sabelko, K.A., K.A. Kelly, M.H. Nahm, A.H. Cross, and J.H. Russell. 1997. Fas and Fas ligand enhance the pathogenesis of experimental allergic encephalomyelitis, but are not essential for immune privilege in the central nervous system. *J Immunol* 159:3096-3099.

Sadelain, M.W., H.Y. Qin, J. Lauzon, and B. Singh. 1990. Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. *Diabetes* 39:583-589.

Sai, P., and A.S. Rivereau. 1996. Prevention of diabetes in the nonobese diabetic mouse by oral immunological treatments. Comparative efficiency of human insulin and two bacterial antigens, lipopolysacharide from Escherichia coli and glycoprotein extract from Klebsiella pneumoniae. *Diabetes & metabolism* 22:341-348.

Sainio-Pollanen, S., S. Erkkila, S. Alanko, A. Hanninen, P. Pollanen, and O. Simell. 1998. The role of Fas ligand in the development of insulitis in nonobese diabetic mice. *Pancreas* 16:154-159.

Sallusto, F., A. Lanzavecchia, and C.R. Mackay. 1998. Chemokines and chemokine receptors in T-cell priming and Th1/Th2-mediated responses. *Immunol Today* 19:568-574.

Sarvetnick, N., D. Liggitt, S.L. Pitts, S.E. Hansen, and T.A. Stewart. 1988. Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. *Cell* 52:773-782.

Sata, M., and K. Walsh. 1998. TNFalpha regulation of Fas ligand expression on the vascular endothelium modulates leukocyte extravasation. *Nat Med* 4:415-420.

Savinov, A.Y., A. Tcherepanov, E.A. Green, R.A. Flavell, and A.V. Chervonsky. 2003. Contribution of Fas to diabetes development. *Proc Natl Acad Sci U S A* 100:628-632.

Schloot, N.C., P. Hanifi-Moghaddam, C. Goebel, S.V. Shatavi, S. Flohe, H. Kolb, and H. Rothe. 2002. Serum IFN-gamma and IL-10 levels are associated with disease progression in non-obese diabetic mice. *Diabetes/metabolism research and reviews* 18:64-70.

Schoenborn, J.R., and C.B. Wilson. 2007. Regulation of interferon-gamma during innate and adaptive immune responses. *Advances in immunology* 96:41-101.

Schram, M.T., N. Chaturvedi, C.G. Schalkwijk, J.H. Fuller, C.D. Stehouwer, and E.P.C.S. Group. 2005. Markers of inflammation are cross-sectionally associated with microvascular complications and cardiovascular disease in type 1 diabetes--the EURODIAB Prospective Complications Study. *Diabetologia* 48:370-378.

Schulthess, F.T., F. Paroni, N.S. Sauter, L. Shu, P. Ribaux, L. Haataja, R.M. Strieter, J. Oberholzer, C.C. King, and K. Maedler. 2009. CXCL10 impairs beta cell function and viability in diabetes through TLR4 signaling. *Cell metabolism* 9:125-139.

Schwenger, G.T., V.A. Mordvinov, and C.J. Sanderson. 2005. Transcription factor GATA-3 is involved in repression of promoter activity of the human interleukin-4 gene. *Biochemistry. Biokhimiia* 70:1065-1069.

Scott, D.E., W.J. Kisch, and A.D. Steinberg. 1993. Studies of T cell deletion and T cell anergy following in vivo administration of SEB to normal and lupus-prone mice. *J Immunol* 150:664-672.

Scriba, T.J., B. Kalsdorf, D.A. Abrahams, F. Isaacs, J. Hofmeister, G. Black, H.Y. Hassan, R.J. Wilkinson, G. Walzl, S.J. Gelderbloem, H. Mahomed, G.D. Hussey, and W.A. Hanekom. 2008. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J Immunol* 180:1962-1970.

Secchiero, P., F. Corallini, M.G. di Iasio, A. Gonelli, E. Barbarotto, and G. Zauli. 2005. TRAIL counteracts the proadhesive activity of inflammatory cytokines in endothelial cells by down-modulating CCL8 and CXCL10 chemokine expression and release. *Blood* 105:3413-3419.

Secchiero, P., C. Zerbinati, E. Rimondi, F. Corallini, D. Milani, V. Grill, G. Forti, S. Capitani, and G. Zauli. 2004. TRAIL promotes the survival, migration and proliferation of vascular smooth muscle cells. *Cellular and molecular life sciences : CMLS* 61:1965-1974.

Seino, K., N. Kayagaki, K. Takeda, K. Fukao, K. Okumura, and H. Yagita. 1997. Contribution of Fas ligand to T cell-mediated hepatic injury in mice. *Gastroenterology* 113:1315-1322.

Selmi, C., and M.E. Gershwin. 2004. Bacteria and human autoimmunity: the case of primary biliary cirrhosis. *Curr Opin Rheumatol* 16:406-410.

Serrano, F., C.F. Calatayud, M. Blazquez, J. Torres, J.V. Castell, and R. Bort. 2013. Gata4 blocks somatic cell reprogramming by directly repressing Nanog. *Stem cells* 31:71-82.

Serreze, D.V., H.D. Chapman, C.M. Post, E.A. Johnson, W.L. Suarez-Pinzon, and A. Rabinovitch. 2001. Th1 to Th2 cytokine shifts in nonobese diabetic mice: sometimes an outcome, rather than the cause, of diabetes resistance elicited by immunostimulation. *J Immunol* 166:1352-1359.

Serreze, D.V., C.M. Post, H.D. Chapman, E.A. Johnson, B. Lu, and P.B. Rothman. 2000. Interferon-gamma receptor signaling is dispensable in the development of autoimmune type 1 diabetes in NOD mice. *Diabetes* 49:2007-2011.

Severin, M.C., Y. Levy, and Y. Shoenfeld. 2003. Systemic lupus erythematosus and parvovirus B-19: casual coincidence or causative culprit? *Clin Rev Allergy Immunol* 25:41-48.

Sewell, D.L., E.K. Reinke, D.O. Co, L.H. Hogan, R.B. Fritz, M. Sandor, and Z. Fabry. 2003. Infection with Mycobacterium bovis BCG diverts traffic of myelin oligodendroglial glycoprotein autoantigen-specific T cells away from the central nervous system and ameliorates experimental autoimmune encephalomyelitis. *Clinical and diagnostic laboratory immunology* 10:564-572.

Shapira, Y., N. Agmon-Levin, and Y. Shoenfeld. 2010. Defining and analyzing geoepidemiology and human autoimmunity. *Journal of autoimmunity* 34:J168-177.

Sharif, S., G.A. Arreaza, P. Zucker, Q.S. Mi, J. Sondhi, O.V. Naidenko, M. Kronenberg, Y. Koezuka, T.L. Delovitch, J.M. Gombert, M. Leite-De-Moraes, C. Gouarin, R. Zhu, A. Hameg, T. Nakayama, M. Taniguchi, F. Lepault, A. Lehuen, J.F. Bach, and A. Herbelin. 2001. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. *Nat Med* 7:1057-1062.

Shehadeh, N., F. Calcinaro, B.J. Bradley, I. Bruchim, P. Vardi, and K.J. Lafferty. 1994. Effect of adjuvant therapy on development of diabetes in mouse and man. *Lancet* 343:706-707.

Shehadeh, N.N., F. LaRosa, and K.J. Lafferty. 1993. Altered cytokine activity in adjuvant inhibition of autoimmune diabetes. *J Autoimmun* 6:291-300.

Shen, C.R., G. Mazza, F.E. Perry, J.T. Beech, S.J. Thompson, A. Corato, S. Newton, R.N. Barker, and C.J. Elson. 1996. T-helper 1 dominated responses to erythrocyte Band 3 in NZB mice. *Immunology* 89:195-199.

Shen, C.R., A.R. Youssef, A. Devine, L. Bowie, A.M. Hall, D.C. Wraith, C.J. Elson, and R.N. Barker. 2003. Peptides containing a dominant T-cell epitope from red cell band 3 have in vivo immunomodulatory properties in NZB mice with autoimmune hemolytic anemia. *Blood* 102:3800-3806.

Sheridan, J.P., S.A. Marsters, R.M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C.L. Gray, K. Baker, W.I. Wood, A.D. Goddard, P. Godowski, and A. Ashkenazi. 1997. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 277:818-821.

Shi, F.D., M. Flodstrom, B. Balasa, S.H. Kim, K. Van Gunst, J.L. Strominger, S.B. Wilson, and N. Sarvetnick. 2001. Germ line deletion of the CD1 locus exacerbates diabetes in the NOD mouse. *Proc Natl Acad Sci U S A* 98:6777-6782.

Shigihara, T., Y. Oikawa, Y. Kanazawa, Y. Okubo, S. Narumi, T. Saruta, and A. Shimada. 2006. Significance of serum CXCL10/IP-10 level in type 1 diabetes. *Journal of autoimmunity* 26:66-71.

Shigihara, T., A. Shimada, Y. Oikawa, H. Yoneyama, Y. Kanazawa, Y. Okubo, K. Matsushima, E. Yamato, J. Miyazaki, A. Kasuga, T. Saruta, and S. Narumi. 2005. CXCL10 DNA vaccination prevents spontaneous diabetes through enhanced beta cell proliferation in NOD mice. *J Immunol* 175:8401-8408.

Shimada, A., J. Morimoto, K. Kodama, R. Suzuki, Y. Oikawa, O. Funae, A. Kasuga, T. Saruta, and S. Narumi. 2001. Elevated serum IP-10 levels observed in type 1 diabetes. *Diabetes care* 24:510-515.

Shoda, L.K., D.L. Young, S. Ramanujan, C.C. Whiting, M.A. Atkinson, J.A. Bluestone, G.S. Eisenbarth, D. Mathis, A.A. Rossini, S.E. Campbell, R. Kahn, and H.T. Kreuwel. 2005. A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity* 23:115-126.

Shoenfeld, Y., C. Selmi, E. Zimlichman, and M.E. Gershwin. 2008a. The autoimmunologist: geoepidemiology, a new center of gravity, and prime time for autoimmunity. *Journal of autoimmunity* 31:325-330.

Shoenfeld, Y., G. Zandman-Goddard, L. Stojanovich, M. Cutolo, H. Amital, Y. Levy, M. Abu-Shakra, O. Barzilai, Y. Berkun, M. Blank, J.F. de Carvalho, A. Doria, B. Gilburd, U. Katz, I. Krause, P. Langevitz, H. Orbach, V. Pordeus, M. Ram, E. Toubi, and Y. Sherer. 2008b. The mosaic of autoimmunity: hormonal and environmental factors involved in autoimmune diseases--2008. *The Israel Medical Association journal : IMAJ* 10:8-12.

Signore, A., A. Annovazzi, R. Gradini, R. Liddi, and G. Ruberti. 1998. Fas and Fas ligand-mediated apoptosis and its role in autoimmune diabetes. *Diabetes/metabolism reviews* 14:197-206.

Signore, A., A. Annovazzi, E. Procaccini, P.E. Beales, J. Spencer, R. Testi, and G. Ruberti. 1997. CD95 and CD95-ligand expression in endocrine pancreas of NOD, NOR and BALB/c mice. *Diabetologia* 40:1476-1479.

Silman, A.J., A.J. MacGregor, W. Thomson, S. Holligan, D. Carthy, A. Farhan, and W.E. Ollier. 1993. Twin concordance rates for rheumatoid arthritis: results from a nationwide study. *Br J Rheumatol* 32:903-907.

Simon, A.K., O. Williams, J. Mongkolsapaya, B. Jin, X.N. Xu, H. Walczak, and G.R. Screaton. 2001. Tumor necrosis factor-related apoptosis-inducing ligand in T cell development: sensitivity of human thymocytes. *Proc Natl Acad Sci U S A* 98:5158-5163.

Singer, G.G., and A.K. Abbas. 1994. The fas antigen is involved in peripheral but not thymic deletion of T lymphocytes in T cell receptor transgenic mice. *Immunity* 1:365-371.

Singh, S.K., and H.J. Girschick. 2004. Molecular survival strategies of the Lyme disease spirochete Borrelia burgdorferi. *Lancet Infect Dis* 4:575-583.

Skrivarhaug, T., H.J. Bangstad, L.C. Stene, L. Sandvik, K.F. Hanssen, and G. Joner. 2006. Long-term mortality in a nationwide cohort of childhood-onset type 1 diabetic patients in Norway. *Diabetologia* 49:298-305.

Sloka, S., M. Grant, and L.A. Newhook. 2010. The geospatial relation between UV solar radiation and type 1 diabetes in Newfoundland. *Acta diabetologica* 47:73-78.

Smith, C.A., T. Farrah, and R.G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 76:959-962.

Smyth, D., J.D. Cooper, J.E. Collins, J.M. Heward, J.A. Franklyn, J.M. Howson, A. Vella, S. Nutland, H.E. Rance, L. Maier, B.J. Barratt, C. Guja, C. Ionescu-Tirgoviste, D.A. Savage, D.B. Dunger, B. Widmer, D.P. Strachan, S.M. Ring, N. Walker, D.G. Clayton, R.C. Twells, S.C. Gough, and J.A. Todd. 2004. Replication of an association between the lymphoid tyrosine phosphatase locus

(LYP/PTPN22) with type 1 diabetes, and evidence for its role as a general autoimmunity locus. *Diabetes* 53:3020-3023.

Smyth, D.J., J.D. Cooper, R. Bailey, S. Field, O. Burren, L.J. Smink, C. Guja, C. Ionescu-Tirgoviste, B. Widmer, D.B. Dunger, D.A. Savage, N.M. Walker, D.G. Clayton, and J.A. Todd. 2006. A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region. *Nat Genet* 38:617-619.

Smyth, M.J., K. Takeda, Y. Hayakawa, J.J. Peschon, M.R. van den Brink, and H. Yagita. 2003. Nature's TRAIL--on a path to cancer immunotherapy. *Immunity* 18:1-6.

Sneller, M.C., S.E. Straus, E.S. Jaffe, J.S. Jaffe, T.A. Fleisher, M. Stetler-Stevenson, and W. Strober. 1992. A novel lymphoproliferative/autoimmune syndrome resembling murine lpr/gld disease. *J Clin Invest* 90:334-341.

Sobel, D.O., J. Han, J. Williams, J.W. Yoon, H.S. Jun, and B. Ahvazi. 2002. Gamma interferon paradoxically inhibits the development of diabetes in the NOD mouse. *Journal of autoimmunity* 19:129-137.

Song, K., Y. Chen, R. Goke, A. Wilmen, C. Seidel, A. Goke, B. Hilliard, and Y. Chen. 2000. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. *J Exp Med* 191:1095-1104.

Staeva, T.P., L. Chatenoud, R. Insel, and M.A. Atkinson. 2013. Recent lessons learned from prevention and recent-onset type 1 diabetes immunotherapy trials. *Diabetes* 62:9-17.

Stassi, G., R. De Maria, G. Trucco, W. Rudert, R. Testi, A. Galluzzo, C. Giordano, and M. Trucco. 1997. Nitric oxide primes pancreatic beta cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. *J Exp Med* 186:1193-1200.

Steinberg, B.J., P.A. Smathers, K. Frederiksen, and A.D. Steinberg. 1982. Ability of the xid gene to prevent autoimmunity in (NZB X NZW)F1 mice during the course of their natural history, after polyclonal stimulation, or following immunization with DNA. *J Clin Invest* 70:587-597.

Strebel, A., F. Bachmann, M. Wernli, and P. Erb. 2002. Tumor necrosis factor-related, apoptosis-inducing ligand supports growth of mouse mastocytoma

tumors by killing tumor-infiltrating macrophages. *International journal of cancer. Journal international du cancer* 100:627-634.

Su, X., Q. Hu, J.M. Kristan, C. Costa, Y. Shen, D. Gero, L.A. Matis, and Y. Wang. 2000. Significant role for Fas in the pathogenesis of autoimmune diabetes. *J Immunol* 164:2523-2532.

Suarez-Pinzon, W., O. Sorensen, R.C. Bleackley, J.F. Elliott, R.V. Rajotte, and A. Rabinovitch. 1999. Beta-cell destruction in NOD mice correlates with Fas (CD95) expression on beta-cells and proinflammatory cytokine expression in islets. *Diabetes* 48:21-28.

Suliman, A., A. Lam, R. Datta, and R.K. Srivastava. 2001. Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and -independent pathways. *Oncogene* 20:2122-2133.

Sun, T., X. Miao, X. Zhang, W. Tan, P. Xiong, and D. Lin. 2004. Polymorphisms of death pathway genes FAS and FASL in esophageal squamous-cell carcinoma. *Journal of the National Cancer Institute* 96:1030-1036.

Sung, H.H., J.H. Juang, Y.C. Lin, C.H. Kuo, J.T. Hung, A. Chen, D.M. Chang, S.Y. Chang, S.L. Hsieh, and H.K. Sytwu. 2004. Transgenic expression of decoy receptor 3 protects islets from spontaneous and chemical-induced autoimmune destruction in nonobese diabetic mice. *J Exp Med* 199:1143-1151.

Suri, A., B. Calderon, T.J. Esparza, K. Frederick, P. Bittner, and E.R. Unanue. 2006. Immunological reversal of autoimmune diabetes without hematopoietic replacement of beta cells. *Science* 311:1778-1780.

Szkudelski, T. 2001. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. *Physiological research / Academia Scientiarum Bohemoslovaca* 50:537-546.

Taghon, T., M.A. Yui, and E.V. Rothenberg. 2007. Mast cell lineage diversion of T lineage precursors by the essential T cell transcription factor GATA-3. *Nat Immunol* 8:845-855.

Tai, T.S., S.Y. Pai, and I.C. Ho. 2013. GATA-3 regulates the homeostasis and activation of CD8+ T cells. *J Immunol* 190:428-437.

Takahashi, T., M. Tanaka, C.I. Brannan, N.A. Jenkins, N.G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell* 76:969-976.

Takeda, K., Y. Hayakawa, M.J. Smyth, N. Kayagaki, N. Yamaguchi, S. Kakuta, Y. Iwakura, H. Yagita, and K. Okumura. 2001. Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. *Nat Med* 7:94-100.

Takeda, K., Y. Hayakawa, L. Van Kaer, H. Matsuda, H. Yagita, and K. Okumura. 2000. Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc Natl Acad Sci U S A* 97:5498-5503.

Takeda, K., M.J. Smyth, E. Cretney, Y. Hayakawa, N. Kayagaki, H. Yagita, and K. Okumura. 2002. Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J Exp Med* 195:161-169.

Tanaka, S., Y. Nishida, K. Aida, T. Maruyama, A. Shimada, M. Suzuki, H. Shimura, S. Takizawa, M. Takahashi, D. Akiyama, S. Arai-Yamashita, F. Furuya, A. Kawaguchi, M. Kaneshige, R. Katoh, T. Endo, and T. Kobayashi. 2009. Enterovirus infection, CXC chemokine ligand 10 (CXCL10), and CXCR3 circuit: a mechanism of accelerated beta-cell failure in fulminant type 1 diabetes. *Diabetes* 58:2285-2291.

Tassell, S.K., M. Pourshafie, E.L. Wright, M.G. Richmond, and W.W. Barrow. 1992. Modified lymphocyte response to mitogens induced by the lipopeptide fragment derived from Mycobacterium avium serovar-specific glycopeptidolipids. *Infection and immunity* 60:706-711.

Tejada-Simon, M.V., Y.C. Zang, J. Hong, V.M. Rivera, and J.Z. Zhang. 2003. Cross-reactivity with myelin basic protein and human herpesvirus-6 in multiple sclerosis. *Ann Neurol* 53:189-197.

Tekmen, I., D. Ozyurt, C. Pekcetin, and Z. Buldan. 2007. The effect of TRAIL molecule on cell viability in in vitro beta cell culture. *Acta diabetologica* 44:60-64.

Thomas, H.E., W. Irawaty, R. Darwiche, T.C. Brodnicki, P. Santamaria, J. Allison, and T.W. Kay. 2004. IL-1 receptor deficiency slows progression to diabetes in the NOD mouse. *Diabetes* 53:113-121.

Thomas, H.E., M.D. McKenzie, E. Angstetra, P.D. Campbell, and T.W. Kay. 2009. Beta cell apoptosis in diabetes. *Apoptosis : an international journal on programmed cell death* 14:1389-1404.

Thomas, H.E., J.L. Parker, R.D. Schreiber, and T.W. Kay. 1998. IFN-gamma action on pancreatic beta cells causes class I MHC upregulation but not diabetes. *J Clin Invest* 102:1249-1257.

Tian, J., P.V. Lehmann, and D.L. Kaufman. 1997. Determinant spreading of T helper cell 2 (Th2) responses to pancreatic islet autoantigens. *J Exp Med* 186:2039-2043.

Tian, J., D. Zekzer, L. Hanssen, Y. Lu, A. Olcott, and D.L. Kaufman. 2001. Lipopolysaccharide-activated B cells down-regulate Th1 immunity and prevent autoimmune diabetes in nonobese diabetic mice. *J Immunol* 167:1081-1089.

Tirabassi, R.S., D.L. Guberski, E.P. Blankenhorn, J.H. Leif, B.A. Woda, Z. Liu, D. Winans, D.L. Greiner, and J.P. Mordes. 2010. Infection with viruses from several families triggers autoimmune diabetes in LEW*1WR1 rats: prevention of diabetes by maternal immunization. *Diabetes* 59:110-118.

Todd, J.A., J.I. Bell, and H.O. McDevitt. 1987. HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* 329:599-604.

Tomonari, K., S. Fairchild, and O.A. Rosenwasser. 1993. Influence of viral superantigens on V beta- and V alpha-specific positive and negative selection. *Immunological reviews* 131:131-168.

Tracy, S., K.M. Drescher, N.M. Chapman, K.S. Kim, S.D. Carson, S. Pirruccello, P.H. Lane, J.R. Romero, and J.S. Leser. 2002. Toward testing the hypothesis that group B coxsackieviruses (CVB) trigger insulin-dependent diabetes: inoculating nonobese diabetic mice with CVB markedly lowers diabetes incidence. *J Virol* 76:12097-12111.

Trautwein, C., T. Rakemann, N.P. Malek, J. Plumpe, G. Tiegs, and M.P. Manns. 1998. Concanavalin A-induced liver injury triggers hepatocyte proliferation. *J Clin Invest* 101:1960-1969.

Triantafyllopoulou, A., N. Tapinos, and H.M. Moutsopoulos. 2004. Evidence for coxsackievirus infection in primary Sjogren's syndrome. *Arthritis Rheum* 50:2897-2902.

Trudeau, J.D., J.P. Dutz, E. Arany, D.J. Hill, W.E. Fieldus, and D.T. Finegood. 2000. Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? *Diabetes* 49:1-7.

Tudela, P., S. Marti, and J. Bonal. 1992. Systemic lupus erythematosus and vaccination against hepatitis B. *Nephron* 62:236.

Turley, S., L. Poirot, M. Hattori, C. Benoist, and D. Mathis. 2003. Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. *J Exp Med* 198:1527-1537.

Unger, W.W., T. Pearson, J.R. Abreu, S. Laban, A.R. van der Slik, S.M. der Kracht, M.G. Kester, D.V. Serreze, L.D. Shultz, M. Griffioen, J.W. Drijfhout, D.L. Greiner, and B.O. Roep. 2012. Islet-specific CTL cloned from a type 1 diabetes patient cause beta-cell destruction after engraftment into HLA-A2 transgenic NOD/scid/IL2RG null mice. *PloS one* 7:e49213.

Uno, S., A. Imagawa, K. Saisho, K. Okita, H. Iwahashi, T. Hanafusa, and I. Shimomura. 2010. Expression of chemokines, CXC chemokine ligand 10 (CXCL10) and CXCR3 in the inflamed islets of patients with recent-onset autoimmune type 1 diabetes. *Endocrine journal* 57:991-996.

Usui, T., R. Nishikomori, A. Kitani, and W. Strober. 2003. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. *Immunity* 18:415-428.

Valle, A., G.M. Giamporcaro, M. Scavini, A. Stabilini, P. Grogan, E. Bianconi, G. Sebastiani, M. Masini, N. Maugeri, L. Porretti, R. Bonfanti, F. Meschi, M. De Pellegrin, A. Lesma, S. Rossini, L. Piemonti, P. Marchetti, F. Dotta, E. Bosi, and M. Battaglia. 2013. Reduction of circulating neutrophils precedes and accompanies type 1 diabetes. *Diabetes* 62:2072-2077.

Vallois, D., C.H. Grimm, P. Avner, C. Boitard, and U.C. Rogner. 2007. The type 1 diabetes locus Idd6 controls TLR1 expression. *J Immunol* 179:3896-3903.

Van den Steen, P.E., P. Proost, A. Wuyts, J. Van Damme, and G. Opdenakker. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* 96:2673-2681.

Van Den Steen, P.E., A. Wuyts, S.J. Husson, P. Proost, J. Van Damme, and G. Opdenakker. 2003. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-

8 process the chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouse GCP-2/LIX and modulate their physiological activities. *European journal of biochemistry / FEBS* 270:3739-3749.

Varanasi, V., L. Avanesyan, D.M. Schumann, and A.V. Chervonsky. 2012. Cytotoxic mechanisms employed by mouse T cells to destroy pancreatic beta-cells. *Diabetes* 61:2862-2870.

Vence, L., C. Benoist, and D. Mathis. 2004. Fas deficiency prevents type 1 diabetes by inducing hyporesponsiveness in islet beta-cell-reactive T-cells. *Diabetes* 53:2797-2803.

Verdonck, E., C.J. Pfau, M.D. Gonzalez, P.L. Masson, and J.P. Coutelier. 1994. Influence of viral infection on anti-erythrocyte autoantibody response after immunization of mice with rat red blood cells. *Autoimmunity* 17:73-81.

Vignaux, F., and P. Golstein. 1994. Fas-based lymphocyte-mediated cytotoxicity against syngeneic activated lymphocytes: a regulatory pathway? *Eur J Immunol* 24:923-927.

Villunger, A., V.S. Marsden, and A. Strasser. 2003. Efficient T cell receptor-mediated apoptosis in nonobese diabetic mouse thymocytes. *Nat Immunol* 4:717; author reply 718.

Villunger, A., V.S. Marsden, Y. Zhan, M. Erlacher, A.M. Lew, P. Bouillet, S. Berzins, D.I. Godfrey, W.R. Heath, and A. Strasser. 2004. Negative selection of semimature CD4(+)8(-)HSA+ thymocytes requires the BH3-only protein Bim but is independent of death receptor signaling. *Proc Natl Acad Sci U S A* 101:7052-7057.

von Herrath, M.G., and M.B. Oldstone. 1996. Virus-induced autoimmune disease. *Current opinion in immunology* 8:878-885.

von Herrath, M.G., and M.B. Oldstone. 1997. Interferon-gamma is essential for destruction of beta cells and development of insulin-dependent diabetes mellitus. *J Exp Med* 185:531-539.

von Meyenn, F., M. Schaefer, H. Weighardt, S. Bauer, C.J. Kirschning, H. Wagner, and T. Sparwasser. 2006. Toll-like receptor 9 contributes to recognition of Mycobacterium bovis Bacillus Calmette-Guerin by Flt3-ligand generated dendritic cells. *Immunobiology* 211:557-565.

Vujanovic, N.L. 2011. Role of TNF superfamily ligands in innate immunity. *Immunologic research* 50:159-174.

Vukkadapu, S.S., J.M. Belli, K. Ishii, A.G. Jegga, J.J. Hutton, B.J. Aronow, and J.D. Katz. 2005. Dynamic interaction between T cell-mediated beta-cell damage and beta-cell repair in the run up to autoimmune diabetes of the NOD mouse. *Physiological genomics* 21:201-211.

Walczak, H., M.A. Degli-Esposti, R.S. Johnson, P.J. Smolak, J.Y. Waugh, N. Boiani, M.S. Timour, M.J. Gerhart, K.A. Schooley, C.A. Smith, R.G. Goodwin, and C.T. Rauch. 1997. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *The EMBO journal* 16:5386-5397.

Waldner, H., R.A. Sobel, E. Howard, and V.K. Kuchroo. 1997. Fas- and FasL-deficient mice are resistant to induction of autoimmune encephalomyelitis. *J Immunol* 159:3100-3103.

Walsh, C.M., B.G. Wen, A.M. Chinnaiyan, K. O'Rourke, V.M. Dixit, and S.M. Hedrick. 1998. A role for FADD in T cell activation and development. *Immunity* 8:439-449.

Wang, B., I. Andre, A. Gonzalez, J.D. Katz, M. Aguet, C. Benoist, and D. Mathis. 1997. Interferon-gamma impacts at multiple points during the progression of autoimmune diabetes. *Proc Natl Acad Sci U S A* 94:13844-13849.

Wang, S.H., Z. Cao, J.M. Wolf, M. Van Antwerp, and J.R. Baker, Jr. 2005a. Death ligand tumor necrosis factor-related apoptosis-inducing ligand inhibits experimental autoimmune thyroiditis. *Endocrinology* 146:4721-4726.

Wang, Y., K.C. Quon, D.A. Knee, A. Nesterov, and A.S. Kraft. 2005b. RAS, MYC, and sensitivity to tumor necrosis factor-alpha-related apoptosis-inducing ligand-induced apoptosis. *Cancer research* 65:1615-1616; author reply 1616-1617.

Wang, Z., J. Hong, W. Sun, G. Xu, N. Li, X. Chen, A. Liu, L. Xu, B. Sun, and J.Z. Zhang. 2006. Role of IFN-gamma in induction of Foxp3 and conversion of CD4+ CD25- T cells to CD4+ Tregs. *J Clin Invest* 116:2434-2441.

Wasmuth, H.E., G. Hess, C. Viergutz, H.R. Henrichs, S. Martin, and H. Kolb. 2000. Non-specific viral infections as possible synchronising events of the manifestation of type 1 diabetes. *Diabetes/metabolism research and reviews* 16:177-178.

Watanabe-Fukunaga, R., C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* 356:314-317.

Watanabe, T., Y. Sakai, S. Miyawaki, A. Shimizu, O. Koiwai, and K. Ohno. 1991. A molecular genetic linkage map of mouse chromosome 19, including the lpr, Ly-44, and Tdt genes. *Biochemical genetics* 29:325-335.

Watanabe, Y., E. Watari, I. Matsunaga, K. Hiromatsu, C.C. Dascher, T. Kawashima, Y. Norose, K. Shimizu, H. Takahashi, I. Yano, and M. Sugita. 2006. BCG vaccine elicits both T-cell mediated and humoral immune responses directed against mycobacterial lipid components. *Vaccine* 24:5700-5707.

Watson, M.L., P. D'Eustachio, B.A. Mock, A.D. Steinberg, H.C. Morse, 3rd, R.J. Oakey, T.A. Howard, J.M. Rochelle, and M.F. Seldin. 1992. A linkage map of mouse chromosome 1 using an interspecific cross segregating for the gld autoimmunity mutation. *Mammalian genome : official journal of the International Mammalian Genome Society* 2:158-171.

Weber, S.E., J. Harbertson, E. Godebu, G.A. Mros, R.C. Padrick, B.D. Carson, S.F. Ziegler, and L.M. Bradley. 2006. Adaptive islet-specific regulatory CD4 T cells control autoimmune diabetes and mediate the disappearance of pathogenic Th1 cells in vivo. *J Immunol* 176:4730-4739.

Weckmann, M., A. Collison, J.L. Simpson, M.V. Kopp, P.A. Wark, M.J. Smyth, H. Yagita, K.I. Matthaei, N. Hansbro, B. Whitehead, P.G. Gibson, P.S. Foster, and J. Mattes. 2007. Critical link between TRAIL and CCL20 for the activation of TH2 cells and the expression of allergic airway disease. *Nat Med* 13:1308-1315.

Wei, G., B.J. Abraham, R. Yagi, R. Jothi, K. Cui, S. Sharma, L. Narlikar, D.L. Northrup, Q. Tang, W.E. Paul, J. Zhu, and K. Zhao. 2011. Genome-wide analyses of transcription factor GATA3-mediated gene regulation in distinct T cell types. *Immunity* 35:299-311.

Wei, J., O. Duramad, O.A. Perng, S.L. Reiner, Y.J. Liu, and F.X. Qin. 2007. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* 104:18169-18174.

Weigle, W.O. 1971. Recent observations and concepts in immunological unresponsiveness and autoimmunity. *Clinical and experimental immunology* 9:437-447.

Weiss, M.J., and S.H. Orkin. 1995. GATA transcription factors: key regulators of hematopoiesis. *Experimental hematology* 23:99-107.

Wen, L., R.E. Ley, P.Y. Volchkov, P.B. Stranges, L. Avanesyan, A.C. Stonebraker, C. Hu, F.S. Wong, G.L. Szot, J.A. Bluestone, J.I. Gordon, and A.V. Chervonsky. 2008. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. *Nature* 455:1109-1113.

Werner, A.B., E. de Vries, S.W. Tait, I. Bontjer, and J. Borst. 2002. TRAIL receptor and CD95 signal to mitochondria via FADD, caspase-8/10, Bid, and Bax but differentially regulate events downstream from truncated Bid. *The Journal of biological chemistry* 277:40760-40767.

Wicker, L.S., J.A. Todd, and L.B. Peterson. 1995. Genetic control of autoimmune diabetes in the NOD mouse. *Annu Rev Immunol* 13:179-200.

Wierdl, M., M. Dominska, and T.D. Petes. 1997. Microsatellite instability in yeast: dependence on the length of the microsatellite. *Genetics* 146:769-779.

Wilberz, S., H.J. Partke, F. Dagnaes-Hansen, and L. Herberg. 1991. Persistent MHV (mouse hepatitis virus) infection reduces the incidence of diabetes mellitus in non-obese diabetic mice. *Diabetologia* 34:2-5.

Wiley, S.R., K. Schooley, P.J. Smolak, W.S. Din, C.P. Huang, J.K. Nicholl, G.R. Sutherland, T.D. Smith, C. Rauch, C.A. Smith, and et al. 1995. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 3:673-682.

Wilson, S.B., S.C. Kent, K.T. Patton, T. Orban, R.A. Jackson, M. Exley, S. Porcelli, D.A. Schatz, M.A. Atkinson, S.P. Balk, J.L. Strominger, and D.A. Hafler. 1998. Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. *Nature* 391:177-181.

Winchester, R. 1992. Systemic Lupus Erythematosus. In Systemic Lupus Erythematosus. R.G. Lahita, editor Churchill Livingstone, New York. 65-85.

Wingender, G., P. Krebs, B. Beutler, and M. Kronenberg. 2010. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol* 185:2721-2729.

Wolfe, S.A., D.E. Tracey, and C.S. Henney. 1976. Introduction of "natural" killer' cells by BCG. *Nature* 262:584-586.

Wong, F.S., C. Hu, L. Zhang, W. Du, L. Alexopoulou, R.A. Flavell, and L. Wen. 2008. The role of Toll-like receptors 3 and 9 in the development of autoimmune diabetes in NOD mice. *Annals of the New York Academy of Sciences* 1150:146-148.

Wong, F.S., I. Visintin, L. Wen, R.A. Flavell, and C.A. Janeway, Jr. 1996. CD8 T cell clones from young nonobese diabetic (NOD) islets can transfer rapid onset of diabetes in NOD mice in the absence of CD4 cells. *J Exp Med* 183:67-76.

Wu, J., C. Metz, X. Xu, R. Abe, A.W. Gibson, J.C. Edberg, J. Cooke, F. Xie, G.S. Cooper, and R.P. Kimberly. 2003. A novel polymorphic CAAT/enhancer-binding protein beta element in the FasL gene promoter alters Fas ligand expression: a candidate background gene in African American systemic lupus erythematosus patients. *J Immunol* 170:132-138.

Wu, J., T. Zhou, J. He, and J.D. Mountz. 1993. Autoimmune disease in mice due to integration of an endogenous retrovirus in an apoptosis gene. *J Exp Med* 178:461-468.

Wu, J., T. Zhou, J. Zhang, J. He, W.C. Gause, and J.D. Mountz. 1994. Correction of accelerated autoimmune disease by early replacement of the mutated lpr gene with the normal Fas apoptosis gene in the T cells of transgenic MRL-lpr/lpr mice. *Proc Natl Acad Sci U S A* 91:2344-2348.

Wu, Q., Q. Yang, E. Lourenco, H. Sun, and Y. Zhang. 2011. Interferon-lambda1 induces peripheral blood mononuclear cell-derived chemokines secretion in patients with systemic lupus erythematosus: its correlation with disease activity. *Arthritis research & therapy* 13:R88.

Yagi, H., M. Matsumoto, S. Suzuki, R. Misaki, R. Suzuki, S. Makino, and M. Harada. 1991. Possible mechanism of the preventive effect of BCG against diabetes mellitus in NOD mouse. I. Generation of suppressor macrophages in spleen cells of BCG-vaccinated mice. *Cellular immunology* 138:130-141.

Yagi, N., K. Yokono, K. Amano, M. Nagata, K. Tsukamoto, Y. Hasegawa, R. Yoneda, N. Okamoto, H. Moriyama, M. Miki, and et al. 1995. Expression of intercellular adhesion molecule 1 on pancreatic beta-cells accelerates beta-cell

destruction by cytotoxic T-cells in murine autoimmune diabetes. *Diabetes* 44:744-752.

Yang, J., I. Kawamura, H. Zhu, and M. Mitsuyama. 1995. Involvement of natural killer cells in nitric oxide production by spleen cells after stimulation with Mycobacterium bovis BCG. Study of the mechanism of the different abilities of viable and killed BCG. *J Immunol* 155:5728-5735.

Yi, Z., L. Li, A. Garland, Q. He, H. Wang, J.D. Katz, R. Tisch, and B. Wang. 2012. IFN-gamma receptor deficiency prevents diabetes induction by diabetogenic CD4+, but not CD8+, T cells. *Eur J Immunol* 42:2010-2018.

Yolcu, E.S., N. Askenasy, N.P. Singh, S.E. Cherradi, and H. Shirwan. 2002. Cell membrane modification for rapid display of proteins as a novel means of immunomodulation: FasL-decorated cells prevent islet graft rejection. *Immunity* 17:795-808.

Yolcu, E.S., H. Zhao, and H. Shirwan. 2013. Immunomodulation with SA-FasL protein as an effective means of preventing islet allograft rejection in chemically diabetic NOD mice. *Transplantation proceedings* 45:1889-1891.

Yoon, J.W., H.S. Jun, and P. Santamaria. 1998. Cellular and molecular mechanisms for the initiation and progression of beta cell destruction resulting from the collaboration between macrophages and T cells. *Autoimmunity* 27:109-122.

Yu, M., L. Riva, H. Xie, Y. Schindler, T.B. Moran, Y. Cheng, D. Yu, R. Hardison, M.J. Weiss, S.H. Orkin, B.E. Bernstein, E. Fraenkel, and A.B. Cantor. 2009. Insights into GATA-1-mediated gene activation versus repression via genome-wide chromatin occupancy analysis. *Molecular cell* 36:682-695.

Zaccone, P., Z. Fehervari, F.M. Jones, S. Sidobre, M. Kronenberg, D.W. Dunne, and A. Cooke. 2003. Schistosoma mansoni antigens modulate the activity of the innate immune response and prevent onset of type 1 diabetes. *Eur J Immunol* 33:1439-1449.

Zauli, G., B. Toffoli, M.G. di Iasio, C. Celeghini, B. Fabris, and P. Secchiero. 2010. Treatment with recombinant tumor necrosis factor-related apoptosis-inducing ligand alleviates the severity of streptozotocin-induced diabetes. *Diabetes* 59:1261-1265.

Zekzer, D., F.S. Wong, L. Wen, M. Altieri, T. Gurlo, H. von Grafenstein, and R.S. Sherwin. 1997. Inhibition of diabetes by an insulin-reactive CD4 T-cell clone in the nonobese diabetic mouse. *Diabetes* 46:1124-1132.

Zhang, X., X. Miao, T. Sun, W. Tan, S. Qu, P. Xiong, Y. Zhou, and D. Lin. 2005. Functional polymorphisms in cell death pathway genes FAS and FASL contribute to risk of lung cancer. *Journal of medical genetics* 42:479-484.

Zhang, X.R., L.Y. Zhang, S. Devadas, L. Li, A.D. Keegan, and Y.F. Shi. 2003. Reciprocal expression of TRAIL and CD95L in Th1 and Th2 cells: role of apoptosis in T helper subset differentiation. *Cell death and differentiation* 10:203-210.

Zhang, Y., A.S. Lee, A. Shameli, X. Geng, D. Finegood, P. Santamaria, and J.P. Dutz. 2010. TLR9 blockade inhibits activation of diabetogenic CD8+ T cells and delays autoimmune diabetes. *J Immunol* 184:5645-5653.

Zhang, Y., H. Wang, J. Ren, X. Tang, Y. Jing, D. Xing, G. Zhao, Z. Yao, X. Yang, and H. Bai. 2012. IL-17A synergizes with IFN-gamma to upregulate iNOS and NO production and inhibit chlamydial growth. *PloS one* 7:e39214.

Zhao, Z.S., F. Granucci, L. Yeh, P.A. Schaffer, and H. Cantor. 1998. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science* 279:1344-1347.

Zipris, D. 2008. Innate immunity and its role in type 1 diabetes. *Current opinion in endocrinology, diabetes, and obesity* 15:326-331.

Zipris, D., E. Lien, A. Nair, J.X. Xie, D.L. Greiner, J.P. Mordes, and A.A. Rossini. 2007. TLR9-signaling pathways are involved in Kilham rat virus-induced autoimmune diabetes in the biobreeding diabetes-resistant rat. *J Immunol* 178:693-701.

……

APPENDIX A

……

ABBREVIATIONS

Ab	Antibody
AICD	Activation Induced Cell Death
AIHA	Autoimmune Haemolytic Anaemia
Aire	Autoimmune Regulator
ALPS	Autoimmune Lymphoproliferative Syndrome
ANA	Antinuclear Antibodies
ANOVA	One-way Analysis of Variance
APC	Allophycocyanin/ Antigen Presenting Cell
APECED	Autoimmune Polyendocrinopathy-candidiasis EctodermalDystrophy
APS1	Autoimmune Polyendocrinopathy Syndrome type 1
ATP	Adenosine triphosphate
BB	Bio-Breeding
BCG	Bacillus Calmette-Guérin
bp	Base-pair
BSA	Bovine Serum Albumin
CFA	Complete Freund's Adjuvant
CFSE	Carboxyfluorescein Succinimydyl ester
CNS	Central nervous system
CO_2	Carbon dioxide
Con A	Concanavalin A
Cr	Chromium
CTLA-4	Cytotoxic T lymphocyte antigen 4
CVB3	B coxsackievirus 3
CY	Cyclophosphamide
dATP	Deoxyadenosine Triphosphate
DC	Dendritic Cell
dCTP	Deoxycytidine Triphosphate
dGTP	Deoxyguanosine Triphosphate

DN	Double negative
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DP	Double positive (CD4+CD8+) thymocyte
DR	Death Receptor
dTTP	deoxythymidine Triphosphate
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylene Diamine Tetra-Acetic Acid
FACS	Fluorescence Activated Cell Sorter
FASL	Fas Ligand
FCS	Foetal calf serum
FcγR	Fc gamma receptor
FITC	Fluoroscein isothiocyanate
gld	Generalized Lymphoproliferative Disorder
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GN	Glomerulonephritis
HA	Haemolytic Anaemia
HCV	Hepatitis C Virus
HLA	Human Leukocyte Antigen
i.p.	Intraperitoneal
i.v.	Intravenous
IC	Immune Complex
ICAM	Intercellular Adhesion Molecule
IDDM	Human insulin-dependent diabetes mellitus
IFN	Interferon
Ifn- γ^{r2}	Interferon gamma receptor 2
Ig	Immunoglobulin
IL	Interleukin

IP-10	Interferon gamma-induced Protein 10kD
ITP	Idopathic Thrombocytopenic Purpura
JAK	Janus Kinase
Kb	Kilobasepairs
kD	Kilo Dalton
KO	Knock-out
LCMV	Lymphocytic Choriomeningitis virus
Lpr	Lymphoproliferation
LPS	Lipopolysaccharide
LYP	Lymphoid Tyrosine Phosphatase
mAb	monoclonal Antibody
MCP-1	Monocyte Chemoattractant Protein-1
mg	Milligrams
MHC	Major histocompatability complex
MHV	Murine Hepatitis Virus
mL	Mililitre
mM	Milimolar
mRNA	Messenger RNA
MS	Magnetic Separation/ Multiple Sclerosis
MST	Median Survival Time
MTV	Mammary Tumor Virus
NF-κB	Nuclear Factor Kappa-B
NK	Natural Killer cell
NKC	Natural killer complex
NKT	Natural Killer T cell
NOD	Non Obese Diabetic
ns	Not Significant
NZB	New Zealand Black

OVA	Ovalbumin
PAGE	Polyacrylamide Gel Electrophoresis
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PI	Propidium iodide
PRR	Pattern Recognition Receptor
RA	Rheumatoid Arthritis
RBC	Red Blood Cell
RBG	Random Blood Glucose
RNA	Ribonucleic Acid
RPM	Revolutions per minute
RT	Room temperature
SAG	Super Antigen
scRNP	Small cytoplasmic Ribonucleic Particles
SEB	Staphylococcal enterotoxin B superantigen
SEM	Standard Error of Mean
SLE	systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
snRNP	small nuclear Ribonucleic Particles
SP	Single positive
SSLP	Simple sequence length polymorphism
SSR	Simple Sequence Repeats
STAT	Signal Transducer and Activator of Transcription
SZ	Streptozotocin
T1D	Type 1 Diabetes

TCR	T cell receptor
Th	T helper
TNF	Tumor Necrosis Factor
TNFSFL	Tumor Necrosis Factor Superfamily Ligands
TNFSFR	Necrosis Factor Superfamily Receptors
TRAIL	TNF-Related Apoptosis Inducing Ligand
Treg	CD4+CD25+ FoxP3+ regulatory T cell
TRITC	Tetramethyl Rhodamine Isothiocyanate
TUNEL	TdT-mediated dUTP-biotin nick end labelling
UV	Ultraviolet
VNTR	Variable Number Tandem Repeat
WT	Wild-type
XPID	X-linked Polyendocrinopathy Immunodeficiency and Diarrhoea
α-GalCer	α-Galactosylceramide
[γ32P]ATP	[γ32P] Adenosine 5' –triphosphate

……

APPENDIX B

……

BUFFERS AND SOLUTIONS

BUFFER FOR CELL SUSPENSION PREPARATION AND FLOWCYTOMETRY

MACS Buffer

500 ml of 1x PBS

0.5 % (w/v) BSA

2mM EDTA

Mix well. Adjust pH to 7.4. Stored at 4°C

DNA EXTRACTION BUFFERS:

Digest Buffer

Stock	*Final*	*Add*
IM Tris pH8.0	100mM	10ml
0.5M EDTA	5mM	1ml
10% SDS	0.2%	2ml
5M NaCl	200mM	4ml
dH2O	upto 100ml	84ml

Proteinase-K

10mg dissolved in 1ml d H2O

Store at -20°C in aliquots

1. Add 500ul digest buffer to approx 1cm of tail in an eppendorf tube.
2. Add 10ul [10mg/ml] proteinase-K.
3. Incubate tails 56°C at least 3hrs (or O/N).

5.25M Guanidine Thiocyanate Lysis Buffer (For 500 ml)

Working Stock	*Reagent Required*	*Conc*	*Add*
118.2 M	Guanidine Thiocyanate	5.25M	310.28g
1.0M	Tris Hcl pH6.5 (TRIZMA)	10 mM	5 ml
0.5M	EDTA	20.0mM	20 ml
100.00%	Triton X100	4.00%	20 ml
154.3 MW	Dithiothreitol (DTT)	64.8mM	5g
	Sterile Milli Q Water		Balance

1. Add GITC, Tris & EDTA bring to 400ml with sterile Milli Q water in a beaker
2. On a heated stirrer @ 55^0C stir until completely dissolved
3. Add DTT then Triton X100 stir gently
4. Allow to cool to RT
5. Place Back on stirrer and adjust pH to 6.5 with HCl
6. Adjust buffer to 500ml with sterile MiliQ water in a graduated 500ml flask

1X Propanol wash Buffer (For 500.0ml)

Working Stock	*Reagent Required*	*Conc*	*Add*
58.4 MW	Sodium Chloride (NaCl)	100.0mM	2.92g
0.5M	Tris Hcl pH8>0 (TRIZMA)	10.0mM	10 ml
100.00%	Isopropanol	25.00%	125 ml
100.00%	Ethyl Alcohol (Ethanol)	25.00%	125 ml
	Sterile Milli Q Water		Balance

1. To 250ml flask add Tris and NaCl, bring to 250ml with sterile Milli Q Water
2. Pour into a 500ml schott bottle
3. Measure in a graduated flask individually, the required volume of both Ethanol and Isopropanol and add to Tris NaCl solution

BUFFERS FOR GEL ELECTROPHORESIS

6 X Gel loading buffer with glycerol

1. Dissolve 25 mg bromophenol blue
2. Add 3 ml glycerol.
3. Make up volume to 10 ml with distilled water.
4. Store at 4°C.

50x TAE Buffer (Tris-Acetate-EDTA)

242 gm Tris base

57.1 ml Acetic acid

100ml 0.5M EDTA

1. Weigh out 242 g Tris base (FW=121.14) and dissolve in 750 mL deionized water.
2. Carefully add 57.1 ml glacial acid acid and 100 mL of 0.5 M EDTA (pH 8.0) and adjust the solution to a final volume of 1 L.
3. This stock solution can be stored at room temperature. The pH of this buffer is not adjusted and should be about 8.5.

1x TAE Buffer (working solution)

The working solution of 1x TAE buffer is made by simply diluting the stock solution by 50x in deionized water. Final solute concentrations are 40mM Tris acetate and 1mM EDTA. The buffer is now ready for use in agarose gel electrophoresis.

……

APPENDIX C

……

COMMUNICATIONS ARISING FROM THIS WORK

Invited talks and posters at conferences

Annual Meeting of the Australian Society for Immunology, 2009, Perth

Annual Meeting of the Japanese Society for Immunology, 2008, Kyoto (Talk)

Annual Meeting of the Australian Society for Immunology, 2008, Canberra (Poster)

Brisbane Immunology Group Annual Retreat, 2007, Sunshine Coast (Talk)

FIMSA/IIS Advanced Immunology Conference, 2006, New Delhi (Talk)

Brisbane Immunology Group Annual Retreat, 2006, Gold Coast (Poster)

Townsville festival of life sciences, 2006, 2007 and 2008 (Poster).

Primary publications

Role of Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) in resistance to Type 1 Diabetes. **Jose R J**, Mysore T B, Silva A, Cretney E, Chowdhury S, Smyth M, Kay TWH, Baxter AG. (*under review: Diabetes*)

Polymorphism in a Fas Ligand simple sequence repeat affects T1D in NOD mice. **Jose RJ,** Walters S, Grey ST, Chowdhury S, Fletcher JM, Gerlach N, Baxter AG. (*manuscript under preparation: Journal of Immunology*)

Role of Ifn-γ in *Mycobacterium bovis* mediated prevention of diabetes, and induction of SLE in NOD mice. **Jose RJ**, Baxter AG (*Manuscript under preparation: International Immunology*)

TNF ligand superfamily members affect NKT cell numbers in NOD mice. **Jose RJ,** Baxter AG (*Manuscript under preparation: Immunology and cell biology)*

Other publications

Urocortin 2 is associated with abdominal aortic aneurysm and mediates anti-proliferative effects on vascular smooth muscle cells via corticotrophin releasing factor receptor 2. Emeto TI, Moxon JV, Biros E, Rush CM, Clancy P, Woodward L, Moran CS, **Jose RJ**, Nguyen T, Walker PJ, Golledge J.Clinical Science. 2013 (IF: 4.86)

Everolimus Limits Aortic Aneurysm in the Apolipoprotein E-Deficient Mouse by Downregulating C-C Chemokine Receptor 2 Positive Monocytes. Moran CS, **Jose RJ**, Moxon JV, Roomberg A, Norman PE, Rush C, Körner H, Golledge J. Arteriosclerosis Thrombosis and Vascular Biology. 2013 Apr;33(4):814-21. (IF: 6.37).

Role of SLAM in NKT cell development revealed by transgenic complementation in NOD mice. NOD.SLAM. Jordan MA, Fletcher JM, **Jose R**, Chowdhury S, Gerlach N, Allison J, Baxter AG. Journal of Immunology. 2011 Apr 1;186(7):3953-65. (IF 5.52)

Herpes simplex virus-1--associated congenital cataract. Raghu H, Subhan S, **Jose RJ**, Gangopadhyay N, Bhende J, Sharma S. American Journal of Ophthalmology. 2004 Aug;138(2):313-4. (IF: 4.22).

Diagnosis of herpes simplex virus-1 keratitis: comparison of Giemsa stain, immunofluorescence assay and polymerase chain reaction. Subhan S, **Jose RJ**, Duggirala A, Hari R, Krishna P, Reddy S, Sharma S. Current Eye Research. 2004. Aug-Sep;29(2-3):209-13. (IF:1.28)

Awards

Research Infrastructure Block grant: JCU internal research project funding, 2011

Young Scientist Research Travel Grant (JDRF): JSI annual conference Kyoto, 2008.

Robert Logan memorial grant: ASI annual conference, Canberra, 2008.

Graduate Research Scheme: JCU internal student research grant, 2007and 2009.

FIMSA travel Bursary: FIMSA/IIS Advanced Immunology course, New Delhi 2006.

International Postgraduate Research Scholarship, JCU 2005