Dissociation of interferon-gamma production and resistance to leishmaniasis in the absence of tumor necrosis factor.

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Chapter 2 The role of TNF in parasitic diseases: Still more questions than answers.

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Declaration of Ethics

The research presented in this thesis was conducted within the guidelines of the James Cook University Statement and Guidelines on Research Practices which is based on the NHMRC Australian Code for the Responsible Conduct of Research (2007). The proposed research methodology received approval from the James Cook University Animal Ethics Committee (A1170 and A1492).

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Abbreviations

AICD	Activation induced cell death
B6	C57BL/6
CBA	Cytometric bead array
cDC	Conventional dendritic cell
ECM	Experimental Cerebral Malaria
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme linked immunosorbent assay
FoxP3	Forkhead box P3
Gata3	GATA binding protein 3
H2	Histocompatability 2
IFNγ	Interferon gamma
IL-4	Interleukin 4 protein
Il-4	Interleukin 4 gene-
IL-10	Interleukin 10 protein
Il10	Interleukin -10 gene

IL-17A	Interleukin 17 A protein
Il-17A	Interleukin 17 A gene
iNOS	Inducible Nitric Oxide Synthase
kDa	KiloDalton
LACK	*Leishmania* homolog of receptor for activated c- kinase
LN	Lymph node
LTα	Lymphotoxin alpha
LTβ	Lymphotoxin beta
MHC	Major Histocompatability Complex
Mo-DC	Monocyte derived Dendritic Cell
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NO	Nitric Oxide
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
pLN	Popliteal lymph node

Rorc	See RorT
RorγT	retinoic acid receptor-related orphan receptor gamma
Tbet	See Tbx21
Tbx21	T box family of transcription factors
TCR	T cell receptor
Th1	T helper 1
Th2	T helper 2
TIM	TNF receptor-associated factor interacting motifs
Tnf	Tumor necrosis factor - gene
TNF	Tumor necrosis factor - protein
TNFR1	TNF receptor 1 (p55,p60)
TNFR2	TNF receptor 2 (p75, p80)

Abstract

The delineation of T helper 1(Th1) and T helper 2 (Th2) responses in promoting resistance and susceptibility to experimental cutaneous leishmaniasis has provided a substantial contribution to the understanding of the molecular basis of T cell differentiation in the context of infectious disease. Dysregulation of these processes renders the host susceptible to disease pathogenesis or immuno-pathology. Yet, the paradigm of resistance and susceptibility fails if the adaptive immune systems is not coupled adequately to the innate immune system. The pleiotropic cytokine Tumor necrosis factor (TNF) is involved in numerous aspects of homeostatic and inflammatory processes involved with immune cell function. Dysregulation of TNF production is associated with autoimmune diseases such as Rheumatoid Arthritis, or can render the host susceptible to infectious diseases. The mechanisms however, by which the overproduction of, or the lack of TNF promotes these extreme outcomes is still relatively unknown. Here, I analsysed the genetic contribution of the different major components of the TNF signalling family to elucidate how TNF confers protection to infection with the intracellular protozoan parasite *Leishmania major*.

Co-operative induction of inducible nitric oxide synthase (iNOS) in mononuclear phagocytes by Interferon gamma and TNF provides the basis for an effective immune response to *L. major*. In the absence of TNF the normally resistant C57BL/6 mouse strain develops a fatal visceralising form of leishmaniasis. Protection from this fatal outcome is dependent on the expression of the trans-membrane but not the soluble form of TNF through an interaction with TNFR1, however the mechanism by which this interaction confers protection remains unknown.

Here I demonstrate that this susceptibility to infection does not result from altered $CD4^+$ effector T cell differentiation or inpaired induction of iNOS. T cell activation is greatly increased in the absence of TNF, however enhancement of activation as measured by increased CD44 expression does not reflect positively on the clinical outcome. $CD44^+$ $CD4^+$ T cells from *L. major* infected TNF-deficient mice showed similar transcriptional up-regulation of both *Tbx-21* and *Ifn-γ* compared to WT controls but showed reduced expression of both *Gata-3* and *Il-10* indicating a more polarized T cell response. This was similarly accompanied by increased levels of IFN-γ that was observed locally and systemically in the absence of either TNF or TNFR1. The up-regulation of IFN-γ in both resistant B6.WT and susceptible B6.TNF-deficient mouse strains correlated with the induction of iNOS that was predominantly expressed by infiltrating $CCR2^+$ inflammatory monocytes. Despite equivalent induction of iNOS in both the lesion and draining lymph node, expression of iNOS and location of *L. major* amastigotes showed distinct cellular compartmentalization. While iNOS expression was restricted to $CCR2^+$ inflammatory monocytes, a novel $CD11b^+$, $iNOS^-$, $Ly6G^-$, $Ly6C^{low}$, $CCR2^{low}$ population was observed that was highly parasitised and accumulated exclusively in the absence of either TNF or TNFR1 in the draining lymph node. The capacity for these $CD11b^+$, $iNOS^-$, ,$Ly6G^-$, $Ly6C^{low}$, $CCR2^{low}$ cells to become highly parasitised did not result from any intrinsic deficit of TNFR signalling. Rather, mixed bone marrow chimeras showed that this sensitivity to *L. major* parasitism results from external cues generated upstream of monocyte and macrophage activation that renders these cells susceptible to infection.

These data demonstrate a unique role for TNF in the coupling of innate and adaptive immune responses through modulating the development of infiltrating myeloid cells that have different leishmanicidal potentials and reflect a state of susceptibility to intracellular

infection to *L. major* rather than promoting direct leishmanicidal functions *in vivo*.

Manuscripts and Presentations Arising From This Thesis.

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Phillip Fromm, Heinrich Körner. TNF-deficient mice exhibit significant changes in the cell-mediated immune response to *L. major*. -Brisbane Immunology Group Annual Conference- Post Graduate Talk- 2007.

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Chapter 1: General Introduction

Tumor Necrosis Factor: a diverse regulator of immunity.

The Tumor Necrosis Factor (TNF) super-family is a large and diverse group of related cytokines, comprised of 19 different ligands. Members of the TNF super-family, such as TNF have been shown to play important roles in many biological processes including haematopoiesis [1, 2], immune cell function [3, 4], and the spatio-temporal organisation of lymphoid tissues [5, 6]. Further to homeostatic functions, TNF and other members of the TNF super-family have also been linked to pathological conditions in humans [7] including tumorigenesis [8-10], type 2 diabetes mellitus [11, 12] and Rheumatoid arthritis [13], and as such have become a major target of pharmaceutical intervention [14].

The gene encoding TNF is located within the MHC region of chromosome 6 in humans and chromosome 17 in mouse in a cluster of closely linked genes including the related Lymphotoxin (*LT*) α and *LT*β genes, with which it shares about 30% amino acid sequence identity [15, 16]. This highly linked region of the genome is highly polymorphic and has been linked with a number of diseases that exhibit complex genetic traits [17]. Since the MHC region has been linked as a genetic determinant of susceptibility or resistance in both autoimmune and infectious disease, the role of TNF in regulating these disease processes is a subject of intense research.

The study of human diseases using mouse models has provided extraordinary insights

into both Mendelian and complex genetic traits and facilitated the elucidation of the

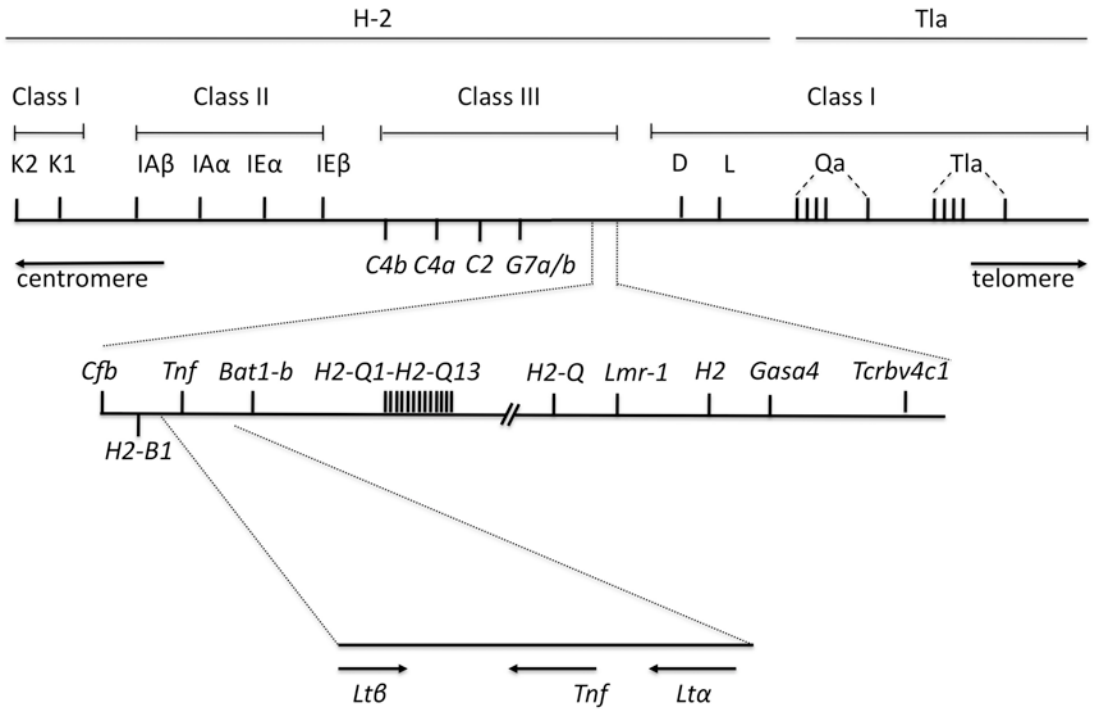

Figure 1.1 Genetic localization of murine *Tnf* within Chromosome 17.
Tnf lies within the H2 locus distal to genes encoding both MHC II and members of the complement cascade. TNF lies directly adjacent to both LTα and LTβ with which it shares sequence homology and is closely linked with a number of immunologically relevant genes including histocompatability genes H2-B1, MHC class 1 like H2Q genes as well as susceptibility loci including *Leishmania resistance gene* 1 (*Lmr-1*) and *Gastritis type A susceptibility locus* (*Gasa4*).

molecular underpinning of health and disease. The high degree of linkage and genetic synteny observed within the MHC however, poses fundamental problems especially when dealing with immune responses. Traditional methods of genetic disruption introduce congenic regions from donor Embrionic stem (ES) cells. The size of these congenic regions subsequently diminish through sequential backcrossing to the parental strain, so that typically after 10 backcrosses less than 0.1% of non linked genes are congenic for the founder ES cell line. However, in the case of TNF the high degree of linkage between other immunologically relevant genes can confound the

Figure 1.2 The TNF super family and its ligands.
The TNF superfamily is comprised of 19 type 2 transmembrane proteins characterized by a conserved 150 amino acid C-terminal TNF homology domain (THD). Many members of the TNF-super family can be cleaved from the cell surface by disintegrin metallopeptidase domain (ADAM), matrilysin or furin family member proteases that yield a soluble ligand. The TNF receptor superfamily displays conserved cysteine rich domains that faciliate binding to THD ligand domains. The number of extracellular N terminal amino acids is shown to the right of each receptor. Reproduced from [7]

interpretation of Mendelian single gene phenotypic interactions due to congenic differences within the MHC region [18]. This has been overcome using ES cells derived from C57BL/6 mice and resulted in the creation of both a B6.TNF-deficient

and a B6.memTNF$^{\Delta/\Delta}$ non-cleavable mutant that does not contain contaminating congenic regions from different mice strains that could influence the immune response [5, 19].

TNF Structure and Function

The Tumor Necrosis Factor (TNF) super-family is a large and diverse group of cytokines comprised of 19 ligands characterised by a conserved 150 amino acid C-terminal homology referred to as the TNF homology domain (THD). This conserved region shares 25-30% sequence identity between family members and is important for the binding to the conserved cysteine rich regions of the extracellular portion of members of the TNF receptor super-family and adapter molecules [20] (See Figure 1.2).

The prototypic family member, TNF, is a pleiotropic type II (extracellular N-terminal) trans-membrane cytokine that is produced by both myeloid [21] and lymphoid cells [22, 23], and is involved in many aspects of immune function [24, 25]. TNF is expressed as a functional membrane bound 26 kDa peptide and undergoes ecto-domain shedding of a 17 kDa subunit following cleavage between residues Ala76 – Val77, primarily by a disintegrin metallopeptidase domain 17 (ADAM 17; also known as TNF alpha converting enzyme) to yield a soluble 17 kDa isoform [26-30].

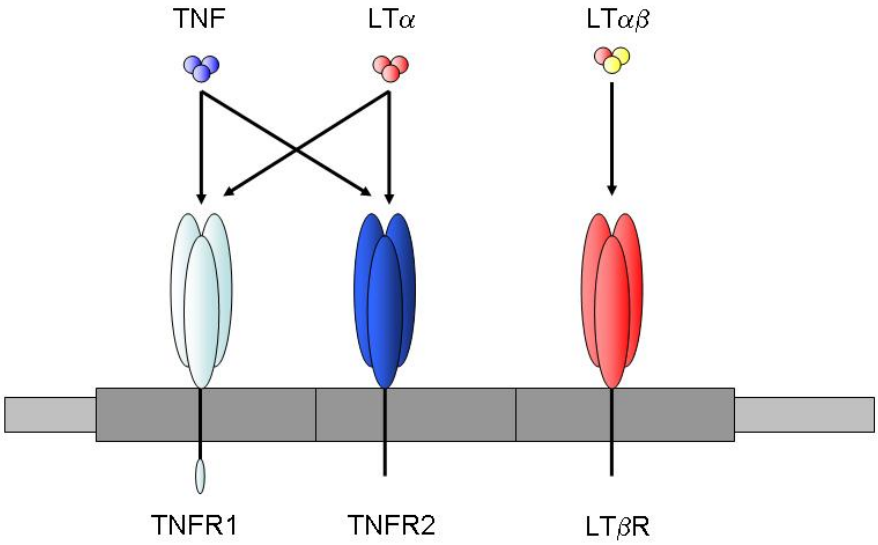

Figure 1.3 TNF exists in membrane bound and soluble isoforms that exert biological effects through two receptors, TNFR1 and TNFR2.
Soluble TNF acts primarily through TNFR1 while membrane bound TNF can act through both TNFR1 and TNFR2. The related LT α homotrimer can also signal through TNFR1 and TNFR2 while LT α1β2 only signals through the LTβ receptor.

Cleavage of membrane TNF by ADAM17 is paralleled by ecto-domain shedding of both TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) to yield soluble isoforms that can modulate inflammation. Sequestration of soluble TNF through competitive binding with soluble receptors as well as reduced cellular avidity for TNF may constitute a mechanism for regulating the biological activity *in vivo* [29-35].

Soluble (sTNF) and transmembrane TNF (memTNF) self assemble to form homotrimers which compete for binding with two distinct differentially expressed trimeric cognate receptors, TNFR1 and TNFR2 [36-39] with the related Lymphotoxin alpha (LTα) homotrimer with which TNF shares 30% amino acid sequence identity (See Figure 1.3). Both the membrane and the soluble forms of TNF exert biological activity through each of its two receptors, to promote a diverse array of responses depending on the cell type. Early biochemical and cellular studies of TNF binding

indicated that soluble TNF had a higher affinity for TNFR1 (K_D $1.9x10^{-11}$M) than TNFR2 (K_D $4.2x10^{-10}$ M) [40] and that memTNF signals preferably through TNFR2 [41]. More biological approaches to address the importance of soluble and membrane TNF were performed using $memTNF^{\Delta/\Delta}$ mice which contain a knocked in Δ1-9,K11E *Tnf* allele [19]. These mice lack soluble forms of TNF but retain normal levels of membrane bound TNF and show that the biological consequences of TNF signalling is only to some extent dependant on the relative affinities of the membrane and soluble ligands for their receptor [19]. Many of the lymphoid structural deficiencies observed in $TNF^{-/-}$ mice are attenuated in $memTNF^{\Delta/\Delta}$ mice, including increased splenic T-B cell segregation, partial restoration of marginal zone metallophilic macrophages and substantial restoration of the marginal sinus [19].

The roles that TNF plays during inflammation are complicated by the duality of the biological processes it activates. TNFR1 is able to promote activation of two functionally divergent signalling pathways that can promote cell survival and activation thorough nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) or alternatively can induce apoptosis through Caspase 8 following proteosomal degradation of TNF receptor associated factor 2 (TRAFF2) and cellular inhibitors of apoptosis (cIAP) [42, 43]. Pre-assembly of the TNFR1 homotrimer followed by binding of soluble or membrane TNF results in mobilization to lipid rafts [44, 45]. Localisation into sphingolipid rich lipid rafts stabilises the formation of the TNFR1 signalling complex and promotes recruitment of TNFR associated death domain (TRADD), receptor-activating protein (RIP) followed by TRAF2 and cIAP1/2 [46]. TRAFF 2 / cIAP1/2 interaction is essential for the induction of canonical NFκB signalling and the inhibition of TNF dependent induced apoptosis [43]. In addition to

anti-apoptotic functions, NFκB activation by TNFR1 signalling serves in this manner to induce the secretion of inflammatory chemokines and cytokines involved in inflammatory responses [47-49].

The initiation of apoptotic signalling through TNFR1 is thought to result from endosomal internalisation of the receptor ligand complex from the lipid raft [46, 50] or through changes in the balance of TRAFF2/cIAP1/2 recruitment to the TNFR1 signalling complex [43] which can be mediated through membrane TNF/TNFR2 [51, 52], resulting in activation of Caspase 8 [50, 53]. Induction of apoptosis through receptor internalisation, unlike other TNF family members such as FAS, only occurs when protein synthesis is blocked or when TNFR1 fails to localise to lipid rafts following activation [44, 50, 53-55].

In contrast to the ubiquitously expressed TNFR1, TNFR2 is expressed predominantly on endothelial cells as well as cells of the immune system [7]. TNFR2 lacks a cytoplasmic death domain characteristic of many members of the TNF super-family but retains TRAF interacting domains (TIMs). This provides potential interaction with TRAFs promoting the induction of NFκB, c-Jun kianses (JNK), extracellular signal regulated kinase (ERK) and Phosphoinosotide-3-kinase (PI3K), that can promote cell proliferation and differentiation [53]. More recently a co-integration of function between TNFR1 and TNFR2 has been proposed that can promote enhanced TNFR1 induced apoptosis [56, 57] through sequestration of NF-κB inducing TRAFF/cIAP complexes [42, 51, 52, 58, 59]. Sequestration of TRAF by TNFR2 allows for promotion of TNFR1 dependent apoptosis through two major mechanisms. Firstly, ubiquitin mediated proteosomal degradation of TRAF2 is mediated by the E3

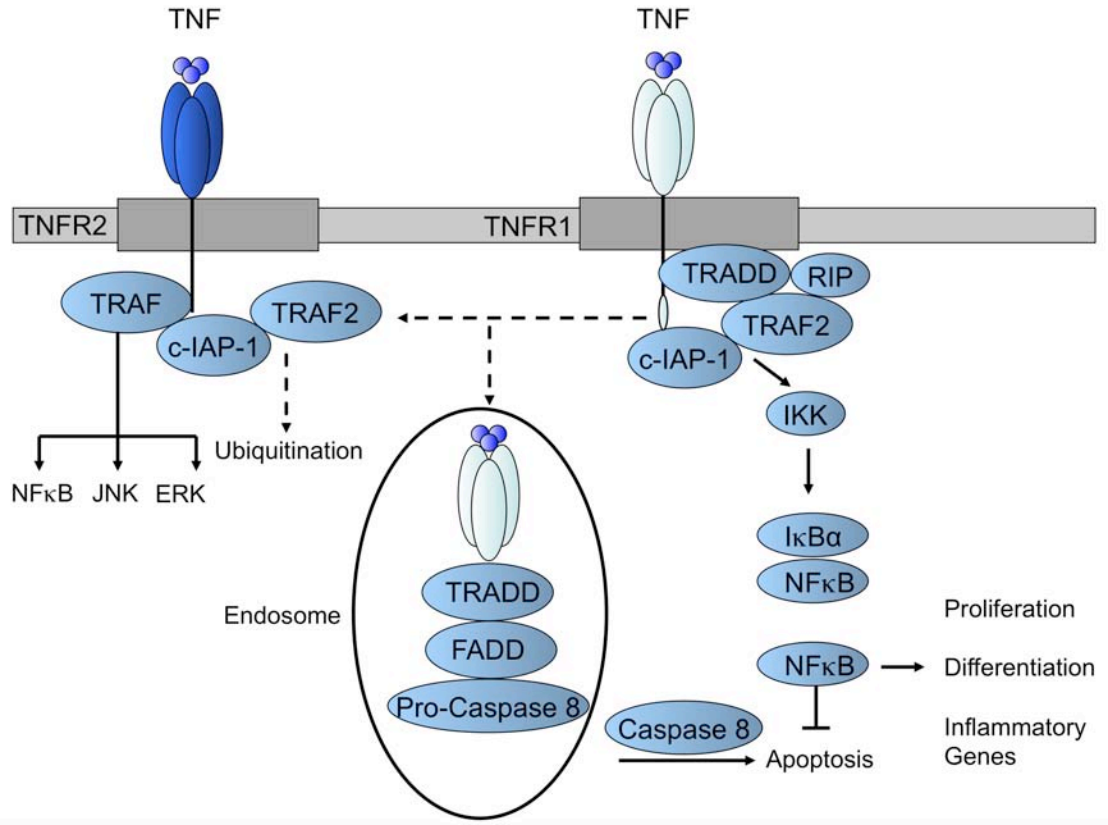

Figure 1.4 Proposed mechanism of TNFR1/ TNFR2 apoptotic crosstalk.
Induction of apoptosis through TNFR1 occurs following internalization of the receptor ligand complex mediated by the co-opting and subsequent ubiqutination of TRAF2 and c-IAP1 following TNF binding to TNFR2. Sequestration of TRAF2 and cIAP inhibits canonical NFκB activation and results in the recruitment and activation of Caspase 8 and internalization of the receptor complex leading to apoptosis. Adapted from [52, 60].

ubiquitin ligase activity of c-IAP1 that constitutes part of both TNFR1 and TNFR2 signalling complex, which prevents induction of NFκB induced survival genes [51, 52]. Secondly, TRAF dependent ubiqitination of RIP1, which provides NFκB independent anti-apoptotic signals is abrogated by means of TRAF sequestration, inducing the alternative NFκB signalling pathway resulting in activation of Caspase 8 [61] (See Figure 2). This activation of the non-canonical p100 NFκB signalling pathway has also been proposed to be able to occur independently of TNFR1, through

a direct interaction between memTNF and TNFR2 [62].

Specialisation of $CD4^+$ T cells.

T helper cells are an integral part of the adaptive immune response that derrive from the thymis expressing both a unique T cell receptor that is able to recognize peptide in the context of MHC II and the co-receptor CD4. Upon activation these CD4+ T helper cells can be broadly divided into functional specilaities resulting from distinct transcriptional prolfiling. This results in in the expression of distinct patterns of cell surface markers and cytokine secretion patterns that imposed by the environement in which they encounter dendritic cells. The first of these to be identified were based upon differential secretion of IFN-γ and IL-4 in $CD4^+$ T cells and were termed Th1 and Th2 respectively [22, 63] and were subsequently shown to to be controlled by the transcription factors Tbet [64, 65] and Gata-3 [66, 67]. The biological consquences of this diversifcation was first highlighted in the disparate outcomes resulting from infection of different strains of laboratory mice with *Leishmania major,* that was directly associated with the production of IFN-γ that resulted in survival or IL-4 that resulted in death [68]. In additional, further T cell subsets have been identified including the Th17 subset that is charactersied by the transcription factror RorγT [69] and the production of the cytokine IL-17, which has been implicated in the immune response to fungal infections but also in chronic inflammatoy autoimmune disease such as multiple sclerosis[70]. In addition to these postive regulatory of the immune system, a T cell subset colliqually refered as a regulatory T cell (Treg) has bee idendtified that is charactersied by the transcription factor FoxP3 and the production of the cytokines TGF-β and IL-10. The deveoplment and lineages of these different

cell subsets are are the subject of much investigation, some degree of plasticity and co regualtion has been observed. For example Gata-3 can directly antagonise development of Th1 cells in an IL-4 independent manner. [71] Similarly, Th17 cells and Tregs share the need for TGF-β in their development despite their distinct functional roles [72].

Regulatory Effects of TNF

In addition to the widespread pro-inflammatory effects induced by TNF, recent evidence point to TNF exerting an anti-inflammatory or regulatory effect that serve to regulate the effector arms of the immune response. A direct inhibitory effect on IL-12 and IL-23 production has been demonstrated in murine macrophages and myeloid derived dendritic cells. This inhibitory effect is only seen following TNF pre-sensitisation prior to exposure to activating stimuli that acts in a TNFR1 dependent manner [73]. Indirect regulation of effector T cell responses at the level of antigen presentation through the actions of TNF on newly differentiated or migrating antigen presenting cells may provide another level of regulation of effector T cells responses.

Regulation of the adaptive immune response can occur as a results of a default inhibitory pathway due to the a reduction in antigen presentation. This can can occur as the immuen resposen reuslts in reducing the titres of the infectious agent Alternatively, regulation can be elicited by means of active suppression through the action of specific regulatory cells. Tregs are a specialised T cell subset that can originate in the thymus (natural Tregs) or can convert from peripheral T effector T cells (induced Tregs) through interaction with dendritic cells [74]. $CD4^+$, $CD25^+$

$GITR^+$ Tregs cells express high level of TNFR2 but little TNFR1 [75]. During the early stages of an immune response TNF can render activated $CD4^+$ T cells refractive to the suppressive effects of regulatory T cells, promoting proliferation [75-77]. TNF also confers direct actions on Treg populations rendering them responsive to TCR ligation and synergising with IL-2, up-regulating expression of the transcription factor FoxP3, which is a hallmark of Treg, and promotes proliferation at the expense of cell contact mediated suppression [75]. This transient suppression of regulatory T cell function in conjunction with increased proliferation potential may provide a temporal basis for immune regulation during the course of infection.

This positive regulation of regulatory function is in contrast to reported studies in human rheumatoid arthritis patients in which TNF plays an important role in the establishment of pathology. The direct pro-inflammatory effects of TNF in disease pathology are paired with changes in the function of Tegs induced by TNF. Treg's from patients with rheumatoid arthritis (RA) fail to suppress $CD4^+$ T cell proliferation and have an impaired ability to suppress IFN γ secretion [78]. This defect in function is pared with phenotypic abnormalities, including increased expression of TNFR2 and reduced FoxP3 expression that can be rescued by neutralising TNF. The failure to suppress effector T cell proliferation and cytokine secretion in RA is ameliorated by treatment with the anti-TNF chimeric antibody Infliximab (Remicade™) (Schering Plough). Neutralisation of TNF in this manner results an overall increase in the numbers of circulating $CD4^+$, $CD25^+$ regulatory T cells that show an increase in FoxP3 expression and restoration of suppressive activity.

Leishmaniasis

Leishmaniasis comprises a spectrum of diseases resulting from infection with facultative intracellular protozoa of the genus *Leishmania* spp. Leishmaniasis in humans can range from a non-fatal cutaneous form (cutaneous leishmaniasis) to a potentially fatal visceral form (visceral leishmaniasis) depending on the strain of *Leishmania* and the immune status of the host.

Figure 1.5 Life cycle of Leishmania major.
L. major promastigotes are transmitted in an anthroponotic or zoonotic manner through the bite of sandfly *Phlebotomus spp.* where they reside in the phagolysosome of resident phagocytes such as macrophages and dendritic cells as replicating amastigotes. Infected cells are taken up in the blood meal of the sand fly where they transform in the midgut into rapidly dividing flagellated promastigotes. Following differentiation into infectious non-dividing metacyclic promastigotes and migration to the salivary glands they are subsequently able to be transmitted in the next blood meal. Reproduced from [79].

The World Health Organisation has classified cutaneous leishmaniasis as a neglected tropical disease [80] since it is invariably non fatal, but results in a high degree of associated morbidity associated with disfigurement. Currently cutaneous leishmaniasis is endemic in 82 countries with approximately 10 million people currently infected. However, due to its prevalence in low socio-ecconomic areas, inconsistency in surveillance and reporting, accompanied with the high cost of medical treatment the reported incidence could be much higher [80-82].

Infection with *L. major* originates naturally through transmission of metacyclic promastigotes from the sand fly host *Phlebotomus papatasi* in a zoonotic or anthroponotic manner where they are able to infect macrophages, dendritic cells and fibroblasts [79, 83, 84]. Cutaneous leishmaniasis is usually self healing following infection with *L. major* or *L. mexicana*, typically resolving after a period of about three months, while infection with *L. tropicana* can take up to a year [85]. While typically self-healing, infection with *Leishmania spp*. is persistent and typically a host maintains parasites for the remainder of its life with the possibility of recrudescence following suppression of the immune system, as is the case after co-infection with HIV [86-88].

Experimental Cutaneous Leishmaniasis.

The intracellular protozoan parasite *Leishmania spp*. has been used extensively to define the paradigm of T cell activation and differential effector phenotypes in response to peptide antigens. Leishmaniasis was a pivotal model in describing the

broad classification of cellular Th1 and humoral Th2 responses [63] resulting from genetic diversity within inbred mouse strains [68].

Resolution of infection is mediated by a strong $CD4^+$ T cell mediated Interferon gamma (IFN-γ) response, the generation of which is dependent on the genetic background of the infected host. Resistant strains of mice including CH3 and C57BL/6 develop strong cellular IFN-γ mediated $CD4^+$ T cell responses, albeit that C57BL/6 mice show a delay in the establishment of this response, while susceptible BALB/c mice develop polarised IL-4 and IL-10 mediated $CD4^+$ T cell responses characteristic of a humoral Th2 response [89].

Table 1.1 Production and Timing of cytokines during leishmaniasis in laboratory mice.

Strain	Cytokine Timing			Susceptibility	Reference
	IL-4	IL-12	IFN-γ		
BALB/c	Early/late	Absent	Minimal	Susceptible	[89, 90]
C3H/HeN	Absent	Maintained	Maintained	Resistant	[89, 90]
C57BL/6	Early	Maintained	Maintained	Resistant	[89, 90]

The disparity observed in the outcomes of different adaptive T cell responses during infection with *L. major* is not solely dependent on the early production of IL-12, which promotes IFN-γ production, or IL-4 to polarise the adaptive T cell response

observed in different inbred mouse strains (See Table 1.1). Resistant C3H/HeN mice show no early IL-4 production while both C57BL/6 and the susceptible BALB/c strains do [90]. While IL-4 production is maintained in the BALB/c mouse, the response becomes dominated by IL-12 and IFN-γ during the course of infection in C57BL/6 mice [89-92].

Despite maintained IL-4 levels in susceptible BALB/c mice, the dominant role of IL-4 in promoting the Th2 paradigm is inconclusive, particularly in light of more recent findings [93]. Treatment using anti-IL-4 mAb (11B11) induces a resistant phenotype with a corresponding induction of IFN-γ. However, concurrent blocking of IFN-γ does not revert the resistance to susceptibility [94, 95]. Experimental cutaneous leishmaniasis in BALB/c mice in the absence of IL-4 or IL-4Rα resulted in complete or partial resistance, respectively, but did not result in a shift in the cellular immune response towards IFN-γ production commonly associated with protection [96]. The disparity observed between IL-4- or IL-4Rα-deficient mice could be attributed to the additional absence of IL-13 signalling in IL-4R$^{-/-}$ mice. These mice present with similar parasite burdens to BALB/c mice although different acute and chronic disease patterns have been noted [97, 98]. Interestingly, BALB/c.IL-13-deficient mice (4 backcrosses) display a resistant phenotype following infection with *L. major* (LV39) [99]. Additional complexity is added when different *L. major* isolates are used for infection of IL-4 or IL-4Rα-deficient mice resulting in a spectrum of clinical outcomes [97]. These mice differ in the control of lesion development, parasite numbers and parasite containment, ranging from a resistant phenotype to a fully susceptible state with necrotising lesions and parasite visceralisation [100].

In contrast to IL-4, IL-10 secretion appears to be imperative for disease progression in an infected host, and serves to promote the concomitant immunity evident in resistant C57BL/6 mice [101]. Opsonisation of *Leishmania* amastigotes, rather than promoting phagocytosis is able to promote FcγR signalling on macrophages and induces IL-10 production that serves to down regulate IL-12, IFN γ, and TNF [102]. Macrophage activation and IL-12 production is inhibited by IL-10 [102], providing an escape from innate effector mechanisms. Additionally IL-10 can serve to directly promote apoptosis of maturing dendritic cells [103-105]. Therefore the pathogens ability in this manner to manipulate IL-10 production by the host, may provide a mechanism for immune evasion that contributes to susceptibility in conjunction with the host's genetic background.

TNF and Experimental Cutaneous Leishmaniasis.

The contribution of the inflammatory cytokine TNF towards the establishment of protective immunity during cutaneous leishmaniasis has been studied extensively and has yielded conflicting results. Early experiments utilising neutralising monoclonal antibodies against TNF appeared to result in the aggravation of the cutaneous lesion [106], while administration of recombinant TNF into resistant CBA, CH3 or susceptible BALB/c mice attenuated lesion development [106, 107] with varying degrees, depdending on whether they displayed a resistant or suceptible genetic background.

Conflicting results began to surface when comparisons were made utilising different

gene knock out strains and transgenic approaches targeted TNF signalling pathways. Similar to experiments using neutralising antibody, transgenic mice bearing a soluble TNFR1-Ig fusion protein developed excacerbated, non-healing lesions following infection [108]. However, gene-targeted mice deficient for TNFR1 or both TNFR1 and TNFR2 developed typical TH1-type responses [109-111], resulting in the control of *L. major* growth but these mice continued to present with a permanent swelling at the site of infection. Furthermore, mice generated on resistant C57BL/6 backgrounds deficient for TNF [5] or mutated to possess a noncleavable membrane form of TNF [19] revealed an important role for membrane TNF. While C57BL/6.TNF$^{-/-}$ (B6.TNF$^{-/-}$) mice succumbed quickly to infection, mice lacking only soluble TNF were able to control the infection similarly to wildtype C57BL/6 (B6.WT) mice [112, 113]. As discussed previously, the location of TNF proximally to the H2D region on murine chromosome 17 makes backcrossing virtually impossible and may confound interpretation of results arising from congenic regions [18]. Utilising mice generated directly in the C57BL/6 background provided an optimal method of studying the contribution of TNF. However, results generated from mice deficient for one or both TNF receptors stills runs contrary to the observations arising using neutralising antibodies or B6.TNF-deficient mice. It is interesting to note that many of these experiments used not only mice generated from different numbers of backcrosses which may explain some of the observed uncertainy, but also different parasite isolates that can also introduce bias into the outcome of infection due to differences in the parasite/host interactions [114]. Ultimately, the reason behind the fatal outcome in *L. major*-infected B6.TNF$^{-/-}$ mice still remains puzzling and remains the focus of this investigation.

Conclusion

The interest in modulating TNF signalling pathways for the purpose of therapeutic intervention has gained widespread use in the treatment of chronic inflammatory conditions, with TNF antagonists such as Etanercept® (Amgen/Wyeth), Infliximab® (Centocor), Adalimumab® (Abbott), Certolizumab®, Golimumab® (Medarex/Centocor) and Tasonermin® (Boerhinger-Ingelheim) having shown great promise in the treatment of inflammatory and autoimmune conditions [14] such as Rheumatoid arthritis, Ankylosing spondylitis, Polyarticular juvenile idiopathic arthritis, Crohn's disease, and Psoriasis [115-117]. However studies are now implicating TNF neutralisation in the recrudescence of existing latent infections, this has been particularly observed with the recrudescence of tuberculosis [117, 118], and more recently leishmaniasis [119-122] in patients undergoing treatment with anti-TNF disease-modifying anti-rheumatic drugs (DMARD's).

The importance of TNF in mediating protection to infection and in regulating other immune responses is well documented [123-125], but the exact mechanisms that govern this protective role are not well described. Additionally, the relationship between TNF induced protection during infection and TNF induced pathology is still the source of much research.

A more comprehensive understanding of the role TNF has in shaping both the innate and adaptive immune responses to intracellular pathogens such as *Leishmania spp*. will provide a greater understanding of the long term consequences of using disease modifying drugs such as TNF antagonists to treat chronic inflammatory diseases.

Thus the experimental aims are as follows:

To determine the role for TNF and its receptors in the generation of a protective immune response to *L. major*.

Examine the role of TNF in shaping T cell responses during infection with *L. major*.

To examine the contribution of TNF on coupling adaptive and innate immune responses against *L. major in vivo.*

Chapter 2: The role of TNF in parasitic diseases: Still more questions than answers

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Abstract

The inhibition of TNF with therapeutic monoclonal antibodies or antibody/receptor fusion proteins in rheumatoid arthritis still constitutes the benchmark for a successful intervention in an ongoing auto-immune-inflammatory disease and underlines the importance of this cytokine. TNF plays a central role in the defence against intracellular infections and is responsible for the promotion of different aspects of the innate immune response such as inflammatory cell recruitment and cell differentiation. While this cytokine generally displays pro-inflammatory activities supporting the early stages of the inflammatory response, it has been demonstrated to be especially important during infection with intracellular pathogens and, consequently, leishmaniasis in $TNF^{-/-}$ mice ends fatally. However, the specific activities of TNF that confer protection are not yet fully understood. This review will summarize the current understanding of TNF function and signalling, and will discuss recent work in the models of malaria, toxoplasmosis, trypanosomiasis and leishmaniasis with particular emphasis on work with gene-deficient mouse models.

Keywords: Tumour necrosis factor, Knockout models, malaria, toxoplasmosis, trypanosomiasis, leishmaniasis

TNF and its two receptors

TNF was first named in 1975 as an endotoxin-induced factor that effected the necrosis of established tumors [126]. The biology of TNF was further characterised over the next 10 years in two parallel lines of research that studied two fundamentally different metabolic activities of this cytokine: its ability to induce cell death in certain transformed cell lines [127, 128] and its suppression of the enzyme lipoprotein lipase A [129, 130]. Initially both activities were ascribed to different molecules: TNF and cachectin. Only after both laboratories had their respective molecules well characterized was their common identity discovered and the name TNF prevailed [131].

TNF is produced predominately by macrophages early after a challenge [21] and is involved in the innate phase of the immune response with a central role in the defence against intracellular pathogens. The cytokine is encoded on chromosome 6 (*Homo sapiens*) or 17 (*Mus musculus*), respectively, in a cluster with the genes encoding lymphotoxin (LT) α and LTβ, two structurally related cytokines [15, 132, 133], and is genetically closely linked to the D region of the major histocompatibility complex (MHC) [16]. It is expressed as a type-II trans-membrane 26 kDa pro-peptide [134, 135] which undergoes shedding of a 17 kDa soluble subunit after cleavage [134] by the matrix-metalloprotease TNF-convertase (TACE, ADAM 17) [28, 30]. Blocking of TNF-convertase inhibits the cleavage of TNF and interferes significantly with the biological activities of the soluble form [27].

TNF has two cognate receptors, TNFR1 (*Tnfrsf1a*, p55, p60) and TNFR2 (*Tnfrsf1b*, p75, p80) [136], which utilize distinct signalling pathways that mediate overlapping biological functions [137, 138]. TNFR1 can promote activation of two functionally divergent signalling pathways which can promote cell survival and activation of pro-inflammatory

mediators thorough nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB) or alternatively, can induce apoptosis through the extrinsic pathway. Differential activation of these pathways appears to be linked to receptor internalisation from lipid raft micro-domains upon ligand binding [139]. TNFR2 has been shown to have its own signalling pathway that can operate distinctly from TNFR1. TNFR2 lacks a cytoplasmic death domain but retains TNF receptor-associated factor interacting motifs (TIMs), which provide potential interaction with TNF receptor-associated factors promoting cell proliferation and differentiation [58, 140].

Soluble and transmembrane TNF-self assemble to form homotrimers [37, 141]. These multimers compete with the related $LT\alpha_3$ homotrimer but not the $LT\alpha_1\beta_2$ heterotrimer for binding to the two distinct and differentially expressed cognate receptors TNFR1 and TNFR2, while $LT\alpha_1\beta_2$ binds to the structurally distinct LTβ receptor [142, 143]. Early *in vitro* studies indicated that soluble TNF interacted with both TNFR1 and TNFR2 and acted preferentially in a pro-inflammatory manner, while trans-membrane TNF had a higher affinity to TNFR2 [41] and provided a co-stimulatory function to B cells [144]. A more targeted biological approach addressing the importance of soluble and trans-membrane TNF has utilised a gene-targeted mouse strain which had been constructed by modifying the cleavage site in the TNF protein [140], thus rendering the proteolytic activity of TACE ineffective ($memTNF^{\Delta/\Delta}$, Δ1-9,K11E TNF allele) [19, 145]. These $memTNF^{\Delta/\Delta}$ mice lack soluble forms of TNF but retain essentially wildtype (WT) levels of transmembrane TNF and show that the biological consequences of TNF signalling are independent of the affinities of the respective ligands for TNFR1 or TNFR2 [19, 145].

Shedding of membrane TNF is paralleled by cleavage of TNFR1 and TNFR2, resulting in

the generation of both membrane-bound and soluble forms of the ligand and its receptors [29]. A study of genetically determined periodic fever syndromes (Familial Hibernian fever, Familial Mediterranean fever) showed that the inflammatory episodes of these syndromes are caused by mutations in the TNFR1 gene that lead to a diminished presence of the soluble receptor in the serum of the patients [146]. Sequestration of soluble TNF through competitive binding with soluble receptors therefore constitutes a central mechanism for regulating the pro-inflammatory activity *in vivo*.

Reverse signalling where TNFR1 or TNFR2 act as the ligand and signals are transduced through membrane TNF could further enhance the complexity of TNF signalling and is still discussed controversially. The transmembrane form of TNF has been reported to posses a casein kinase 1 motif that can be phosphorylated at a conserved serine residue and can result in mobilisation of intracellular calcium [147, 148] and the production of IL-2 and IFN-γ in human CD4 T cells [149].

TNF-deficient animal models: A caveat

In models of parasitic disease, resistance or susceptibility to infection depends to a large extent on the genetic background of the host. For example, genetic linkage studies have identified the H2 locus as a resistance factor to *Leishmania major* [150, 151], to *Toxoplasma gondii* [152] and to *Plasmodium chabaudi* [153]. The H2 locus is a highly polymorphic region containing a range of genes important in regulating immune function such as major histocompatibility antigens as well as components of the complement cascade and pro-inflammatory cytokines such as TNF and LT [18]. The determination of

the exact contribution of TNF in many models of infection has been complicated by the use of knockout models with ill-defined genetic backgrounds (C57BL/6 x 129) and variations in the strain of the infecting parasite [114]. Because TNF is located proximally to the H2D region on murine chromosome 17 and is closely linked to the MHC [15, 16], genetically clean back-crossing becomes almost impossible. Only knockout models established on the correct genetic background provide an optimal tool for the elucidation of the role TNF plays in infection, without complications arising from the presence of large congenic regions in immunologically important areas of the genome [18]. The genetic background of the mouse strains used will therefore be highlighted in the following examples.

Role of the proinflammatory cytokine TNF and its receptors in parasitic diseases

TNF and its receptors in malaria

Symptomatic malaria infection arises when the causative parasite, *Plasmodium* spp., invades and replicates within circulating erythrocytes, frequently causing relatively mild symptoms such as nausea and fever, but occasionally more severe problems such as anaemia, respiratory distress and cerebral malaria (CM). In CM, *Plasmodium falciparum*-infected erythrocytes cyto-adhere to the brain microvasculature, resulting in a massive functional disturbance of the brain that is often life threatening. Furthermore, pregnant women living in holoendemic areas are also susceptible to placental malaria, where

Table 2.1 Contribution of genetic background to immunity to blood-stage and cerebral malaria in TNF/TNF receptor (TNFR) knockout mice.

Genetic Background/ Treatment	***Plasmodium* spp.**	**Clinical Outcome**	**Reference**
C57BL/6	*P. berghei* *P. chabaudi*	Rapidly fatal CM Resolution of blood stage infection	[154, 155] [156-158]
C57BL/6-*Tnf*	*P. berghei* *P. chabaudi*	Develop CM Resolution of blood stage infection	[159, 160] [156]
C57BL/6.CBA–*Tnf* (five generations)	*P. chabaudi*	Increased parasitemia, increased mortality	[161]
TNF Antagonist (CBA strain)	*P. berghei*	Protected from CM	[162]
Thiolated human recombinant TNF	*P.berghei*	Protected from CM	[163]
Recombinant TNF	*P. berghei*	Decreased parasite burden, protected from CM	[163]
Recombinant TNF	*P. chabaudi*	Decreased parasite burden	[164]
Transgenic human TNF	*P. yoeli, P. chabaudi*	Decreased parasite burden	[165]
Transgenic soluble TNFR1-fusion protein	*P. berghei*	Protected from CM	[108]
C57BL/6.129Sv/Ev-*Tnf Ltα*	*P.berghei*	Protected from CM	[166]
C57BL/6- *Ltα*	*P. chabaudi*	Resolution of blood stage infection	[156]
C57BL/6-*Tnfrsf1a* (TNFR1*)*	*P.berghei* *P. chabaudi*	Susceptible to CM Resolution of blood stage malaria	[167-170]
C57BL/6-*Tnfrsf1b* (TNFR2)	*P.berghei*	Protected from CM	[168-170]
C57BL/6.129S– *Tnfrsf1a* T*nfrsf1b* (TNFR1/TNFR2*)*	*P. chabaudi*	Resolution of blood stage malaria	[171]

CM, Cerebral Malaria

parasite sequestration within the placenta leads to local inflammation and often loss of the foetus. The nature and severity of malaria results from an ensuing battle between the replicating parasite and the host response to infection. An appropriate and rapid host response limits parasite growth and therefore symptom severity, resulting in a favourable outcome. Severe forms of the disease occur when a combination of high parasite loads and an exuberant and damaging inflammatory response overwhelm the host. TNF appears to play important roles in both of these clinical courses of the disease.

Production of TNF in malaria is stimulated in circulating monocytes, macrophages and lymphocytes by a number of parasite-derived products that are released during erythrocytic infection [172-175]. High levels of TNF have been detected in the serum of patients suffering from malaria, correlating with disease severity and death [176, 177]. TNF has also been associated specifically with CM [178, 179] [180] and placental malaria [181]. Several studies of genetic polymorphisms within the promoter of the human *Tnf* gene have been associated with disease severity in malaria due to their influence on gene expression and protein levels [182, 183]. The hypothesis that the pathology associated with malaria results from the potentially toxic effects of TNF has motivated the staging of a number of clinical trials using TNF-neutralising monoclonal antibodies or antibody/receptor fusion proteins. To date none have proved effective and in some cases have had detrimental effects on the clinical outcome. For example, a large placebo-controlled trial using a monoclonal anti-TNF antibody that was conducted in Gabonnese children suffering CM resulted in no improvement in survival but an increase in residual neurological sequelae [184]. Thus, the role of TNF in malaria is likely to be more complex than originally appreciated.

Significant advances in our understanding of the role of TNF in malaria have been made from studying mouse models of the disease (experimental CM, ECM), which replicate many aspects of the pathology observed in human CM. The murine-specific parasite strain, *Plasmodium berghei* ANKA, when introduced into susceptible strains of mice, replicates rapidly in the circulation and then causes an overwhelming CM within 7-10 days, characterized by occlusion of cerebral vasculature with infected erythrocytes, T-cell activation and disruption of the blood-brain-barrier [154, 155]. Localised production of TNF appears to be central to the development of ECM. TNF transcript and protein levels are specifically elevated in the brains of ECM-susceptible, but not resistant mice [185, 186]. Early studies also demonstrated that treatment of susceptible CBA mice with neutralising TNF antibodies protected against ECM development [162] and genetic disruption of the TNF locus (in a mixed genetic background) resulted in resistance to ECM [166]. In conjunction with other studies, this led to the hypothesis in which high levels of locally-(brain) produced TNF drive the pathological development of ECM. This mechanism also involves the TNF-mediated up-regulation of adhesion molecules on the brain vascular endothelium (eg. ICAM-1, VCAM-1, E-selectin) promoting, in the murine model, a limited sequestration of parasitised red blood cells (pRBC) and leukocyte accumulation [187]. TNF is also capable of directly disrupting the blood-brain-barrier [188]. Related murine studies indicate similar roles for TNF in placental malaria. TNF production is up-regulated and sustained in the placenta of infected pregnant mice and is associated with inflammation, organ disruption, haemorrhage and foetal loss. Treatment with neutralising TNF antibodies protects against these pathologies [189].

However, recent studies, designed to address a number of problems associated with these earlier murine experiments, have questioned the importance of TNF in ECM. The TNF

locus is genetically linked to LTα, and the MHC regions of the different mouse strains are heterogeneous, potentially influencing the clinical outcomes described (Table 2.1). Therefore, gene-deficient mouse strains were established from C57BL/6 (ES) cells. Infection of TNF and/or LTα-deficient mice (C57BL/6 background) with *P. berghei* ANKA indicated that LTα, but not TNF, was necessary for the development of ECM [159, 160]. Additional studies utilising bone marrow chimeric mice indicated LTα in ECM etiology is derived from a radiation-resistant cell population, possibly the brain endothelium [159]. As already noted by others [190], this raises the need for re-evaluation of TNF in the clinical setting of CM, especially since some of the immunological reagents used to measure serum TNF levels might cross-react with LTα.

Several studies using other murine-specific parasite species such as *P. chabaudi* and *Plasmodium Yoelii,* have been used to investigate the roles of the TNF axis in both the host response and the ensuing pathology associated with malaria. These species typically give rise to an increasing blood parasitemia that peaks in correlation with mouse strain susceptibility but do not cause ECM. Resistant mice limit peak parasitemia and resolve the infection via effective innate defences and appropriately timed development of cellular adaptive immune responses. Susceptible mice suffer higher parasite loads and an inappropriate and sustained Th1-like inflammatory response, resulting in severe anaemia and other systemic metabolic problems that prove fatal [191]. In general, the timing and magnitude of TNF production appears critical in evoking protective or pathogenic effects in these models. Transgenic mice expressing human TNF had reduced parasitemia following infection with either *P. chabaudi* or *P. yoelii* [165]. In contrast, others have shown that susceptible mice display higher levels of circulating TNF than resistant strains at the peak of parasitemia, whereas resistant mice express TNF at earlier stages of

infection, which is subsequently down-regulated [157, 192, 193]. The timing of TNF production in resistant mice is associated with an early Th1 response during *P. chabaudi* infection [157]. Administration of neutralising TNF antibodies to susceptible mice provides significant protection against mortality [158, 194, 195], whereas similarly treated resistant mice display increased susceptibility [157]. Treatments with neutralizing antibodies had no dramatic effects on parasitemia in either scenario but instead, influenced subsequent pathology. For example the liver damage observed in susceptible DBA2/J mice was eliminated by anti-TNF treatment [194]. In agreement with these results, administration of low doses of recombinant human TNF also reduced parasite growth in the ECM model in *P. berghei*-infected mice, whereas higher doses of TNF accentuated the cerebral pathology [163].

The infection of C57BL/6 mice with *P. chabaudi* normally results in a resolution of the blood-stage of malaria, whereas *P. chabaudi* infection of different knockout strains of mice genetically deficient for TNF have yielded conflicting results. Increased parasitemia and mortality was noted in *P. chabaudi*-infected C57BL/6.CBA–*Tnf* mice (BL/6 x CBA-derived ES cells) [161], consistent with a role for the cytokine in protection against parasite growth. In contrast, C57BL/6-*Tnf* and *Lt*α-deficient mice established on a pure genetic background (C57BL/6-derived ES cells) were able to clear parasites and survive an infection similar to WT C57BL/6 animals. Interestingly, the C57BL/6.*Lt*α-deficient mice showed delayed IFNγ and TNF production, indicative of an immunomodulatory role for LTα [156].

A further level of complexity is the regulation of TNF by the TNF family of receptors, including TNFR1, TNFR2 and LTβR. Soluble forms of these molecules are found at high

concentrations in plasma of malaria sufferers, often in association with disease severity and high TNF levels [196, 197]. Since these molecules are capable of down-regulating TNF activity, they have been hypothesised to act in a counter-regulatory and possibly beneficial fashion in malaria. A number of murine malaria studies support this hypothesis, although there are also inconsistencies. Following infection with *P. chabaudi* TNFR1-deficient animals showed increased levels of the gametocyte form of the parasite, thereby enhancing transmission of the infection via the mosquito vector and were unable to develop an effective adaptive memory-based response upon re-challenge with *P. chabaudi* [167, 171, 198]. In contrast, TNFR1 had a protective effect in the development of *P. berghei*-induced ECM when expressed transgenically as a soluble molecule [108]. However, establishment of *P. berghei*-induced ECM is still observed in mice (pure C57BL/6 background) deficient for TNFR1 or LTβR, but not in the absence of TNFR2, which is expressed on brain endothelium during ECM in WT mice, indicating a pathological role for TNFR2 [168-170, 199, 200]. This is supported in the *P. chabaudi* model in mice lacking both TNF receptors (on a mixed genetic background), which showed no marked difference to WT animals in clearing parasites. Counter-intuitively, high levels of soluble TNFR2 have recently been found in Indonesian patients with severe malaria, in conjunction with an expanded TNFR2-positive T-regulatory cell subset. This would implicate an immunosuppressive role for TNFR2 [201], shedding a different light on the role of TNFR2 in ECM. However, a similar study in an African population failed to replicate this correlation, suggesting that additional genetic complexities are involved [202]. Taken together, there appears to be a number of variables that influence the pathogenic versus protective potential of TNF and LTα and their receptors, including the timing, level and location of the production of these molecules, as well as more complex

host pathogen-specific factors.

The role of TNF and TNF receptors in toxoplasmosis

The obligate intracellular protozoan parasite *T. gondii* infects approximately one-third of the human population [203]. Following oral infection, the parasite passes the intestinal barrier, disseminates in macrophages and dendritic cells within the host and infects various lymphatic and parenchymal organs including the CNS. In response to the infection, a protective T cell response is established, which prevents the death of the host by parasite-induced tissue necrosis. However, the parasite evades complete elimination by the immune response due to intraneuronal persistence [204]. If T cell immunity to *T. gondii* wanes, as observed in AIDS patients, intra-neuronal *T. gondii* cysts can be reactivated and result in necrotizing toxoplasmic encephalitis (TE), which can prove lethal [205, 206].

In toxoplasmosis, various cell types including macrophages, microglia, neutrophils, T cells and dendritic cells produce TNF. Production of TNF is induced by IFN-γ in infected cells and the latter cytokine and its receptor have a pivotal role in the control of *T. gondii* in mice [207]. In addition to IFN-γ, *T. gondii* glycosylphosphatidyinositols can directly induce TNF production in macrophages via a MyD88-dependent pathway [208]. On the other hand, fatty acids of *T. gondii* inhibit TNF production in macrophages, which could represent one strategy of the parasite to evade eradication by the immune system [208]. The assumption that *T. gondii* actively inhibits TNF production of myeloid cells is further supported by the finding that infection of neutrophils with *T. gondii* inhibits TNF cell surface expression induced by lipopolysaccharide treatment [209]. Furthermore, *T. gondii* actively inhibits the translocation of the transcription factors STAT1 and NF-κB to the

Table 2.2 Contribution of genetic background to immunity to toxoplasmosis in TNF family knockout mice.

Genetic Background/Treatment	*Toxoplasma* spp.	Clinical Outcome	Reference
BALB/c	*T. gondii*	Efficient control of acute and chronic TE	[211]
C57BL/6	*T. gondii*	Chronic progressive TE	[212]
anti-TNF-treated C57BL/6J	*T. gondii*	Lethal acute Toxoplasmosis	[212]
anti-TNF-treated C57BL/6J x BALB/c (CB6F1)	*T. gondii*	No increased mortality	[212]
TNF mAb treatment in chronic TE of C57BL/6 mice	*T. gondii*	Lethal reactivation of chronic TE	[213]
TNFR1 (B6 x 129 random background)	*T. gondii*	Acute lethal TE, increased parasite burden	[214]
TNFR2 (B6 x 129 random background)	*T. gondii*	Survival of acute TE, normal parasite control	[214]
TNFR1/2 (B6 x 129 random background)	*T. gondii*	Acute lethal TE, increased parasite burden	[214]
C57BL/6 *Tnf*	*T. gondii*	Acute lethal TE, increased parasite burden	[215]
C57BL/6 *Lt*α	*T. gondii*	Acute lethal TE, increased parasite burden	[215]
C57BL/6 *Tnf Lt*α	*T. gondii*	Acute lethal TE, increased parasite burden	[215]

TE, Toxoplasmic encephalitis; LT, lymphotoxin; mAb, monoclonal antibody.

nucleus which, in turn, results in a suppression of TNF production [210]

Mouse models of toxoplasmosis have been extremely useful in analyzing and understanding different aspects of the immune response to *T. gondii*. This is mainly due to the fact that (i) murine TE is induced upon oral infection, i.e. the natural route of infection, (ii) mice are a natural host of *T. gondii* and (iii) mice develop an acute TE followed by a chronic stage with parasite persistence similar to the natural course of human disease. Acute TE starts approximately 7 days after oral infection and is characterized by the

infection of multiple brain parenchymal cell types including microglia, astrocytes and neurons as well as the recruitment of inflammatory leukocytes including CD4 and CD8 T cells, macrophages and granulocytes. Acute TE is phenotypically largely identical in all mouse strains but the course of chronic TE is strongly determined by the host genetic background [152, 216]. In BALB/c mice, the parasite load starts to decline around day 30 p.i. and these animals survive chronic TE without limitation of their lifespan. In contrast, C57BL/6 mice develop a chronic progressive TE and ultimately succumb to the disease after 3-4 months. Genetic studies initially suggested that the TNF locus determines resistance to TE in mice [217]. However, later studies revealed that both the MHC haplotype and non-MHC-linked genes contribute to susceptibility to TE [216] and that the MHC class I L^d gene confers resistance in BALB/c mice [211] These data clearly emphasise that genetic studies on TNF have to be carefully structured to avoid misinterpretation due to the close linkage of *Tnf* to the H2 complex.

Experimental therapy using a neutralizing TNF monoclonal antibody (mAb) was sufficient to reactivate TE in chronically infected C57BL/6 mice [213] In these animals chronic TE was associated with a severe persisting meningoencephalitis and disruption of the blood-brain barrier. In infection experiments using C57BL/6-*Tnf* mice (see Table 2.2) a normal parasite-specific T cell response was mounted. However, T cell-derived IFN-γ was insufficient to induce normal intracerebral inductible nitric oxide synthase (iNOS) production by TNF-deficient macrophages, which is crucial for the control of the infection [215]. Consequently, this resulted in a lethal acute necrotizing TE, which was characterized by an insufficient control of parasite replication and, ultimately, the death of $TNF^{-/-}$ mice at approximately day 27 [215] Thus, TNF is essential to control both acute and chronic murine TE. In contrast to the CNS, TNF was not required for the control of

the parasite in other organs indicating that TNF-deficiency can be compensated for in toxoplasmosis [215]. Furthermore, earlier studies in genetically modified mice dissected the usage of the two TNFR signaling pathways in the response to *T. gondii* infection and revealed that TNFR1 but not TNFR2 is required for control and survival of TE [214]. Furthermore, it has been demonstrated that in addition to TNF, LTα is essential for the intracerebral control of *T. gondii* and the survival of acute TE [215]. Additionally, it has been reported that stimulation of *T. gondii*-infected mice via CD40 reduced the *in vivo* parasitic load through a TNFR2-dependent mechanism [218]. Additional analysis of reciprocal bone marrow chimeras with C57BL/6-*Tnf* and C57BL/6 as well as TNFR1/2$^{-/-}$ and C57BL/6 mice demonstrated that hemopoitic cells have to produce TNF, whereas both hemopoitic and (brain) parenchymal cells have to express TNFRs in order to control TE [214, 215, 219]

In humans, studies in rheumatoid arthritis patients treated with TNF antagonists have revealed that in rare cases ocular toxoplasmosis may be induced by TNF blockade, although this complication of anti-TNF therapy is much less common then the reactivation of tuberculosis [220]. Treatment with TNF antagonists does not reactivate TE, which may be explained by the inability of the antibody to pass the blood-brain barrier [203]. Interestingly, the reactivation of *T. gondii* in the CNS is largely restricted to AIDS patients and rarely observed in other immuno-deficient patients.

Collectively, these findings illustrate the central protective role of TNF in toxoplasmosis and, not surprisingly, show that *T. gondii* has developed several immune evasion strategies against TNF induction in order to enable its persistence in the host.

TNF in experimental trypanosomiasis

Sleeping sickness (human African trypanosomiasis) and Chagas' disease (American trypanosomiasis) are two important diseases caused by the parasitic protozoa of the genus *Trypanosoma*, in sub-Saharan Africa and South America, respectively [221]. African sleeping sickness presents with a spectrum of manifestations ranging from a chronic form following infection with *Trypanosoma brucei gambiense* that, after a short systemic stage, progresses inadvertently into a central nervous syndrome that can last years, to an acute form after infection with *Trypanosoma brucei rhodesiense*, which results in an acute infection. Both forms are fatal without intervention and can only be treated with chemotherapy [222]. In contrast, South American Chagas disease caused by infection with *Trypanosoma cruzi* presents biphasically with an initial acute phase followed by a chronic, predominantly subclinical, phase in which 20-40% of patients eventually develop myocardial lesions that result in severe cardiomyophathy [223].

The parasites can reside in all nucleated cells but the main targets in the infective acute stage are macrophages [224]. The *Trypanosoma*-specific activation of macrophages is mediated by glycosylphosphatidylinositol anchors of specific *T. brucei* [225] and *T. cruzi* surface glycoproteins [226]. A hallmark of the cellular response in macrophages against both *T. cruzi* [227] and *T. brucei* [228] is the up-regulation of TNF, which can, in the presence of IFN-γ, be correlated with nitric oxide-mediated killing of the parasites [227]. Treatment with anti-TNF antibodies inhibits trypanocidal activity in macrophages [229]. Interestingly, it could be demonstrated that a direct trypanocidal effect of TNF on *T. brucei* exists [230]. A neutralization of this activity by either soluble TNFR1 or TNFR2 did not occur, but an inhibition by N,N'-diacetylchitobiose, an oligosaccharide that binds TNF,

could be shown [231].

Due to the complexity of the pathology, controversies and misinterpretations regarding the roles of TNF in the human systemic response are commonplace. Therefore, to better understand TNF function in trypanosomiasis, different rodent models have been employed (Table 2.3).

The first *in vivo* treatment of susceptible BALB/c mice with recombinant TNF unexpectedly demonstrated an exacerbated disease that resulted in a more acute course of disease after infection with *T. cruzi* [232]. A series of subsequent infection experiments used anti-TNF antibodies to further elucidate the relationship between the presence of TNF and host resistance. Injection of the IgM anti-TNF antibody IF3F3 in *T. cruzi*-infected BALB/c mice resulted in an attenuation of cachexia if given early in the infection [233]. In the same experimental system a dichotomy in the response to different anti-TNF mAbs was observed. While the rat mAb TN3 increased circulating TNF, worsened cachexia and increased mortality, the anti-TNF mAb 1F3F3 was protective to some extent [234]. Both antibodies are neutralising and the observed differences are difficult to reconcile but differences in the pharmacogenetics of an IgM versus an IgG antibody or, more unlikely, an interference with the shedding of soluble TNFR2, which is protective, could be an explanation [234, 235].

Infection experiments using gene-deficient mouse strains further emphasised the important role of TNF (Table 2.3). Infection of a TNFR1-deficient mouse strain (back-crossed 9 x to C57BL/6) with *T. cruzi* resulted in higher parasitemia and increased mortality. This coincided with increased immunopathology as measured by necrotic lesions in muscles. The mice produced normal levels of nitric oxide in response to the infection but were

deficient in the production of *Trypanosoma*-specific IgG [236]. The infection of $TNF^{-/-}$ mice (mixed background, back-crossed to C57BL/6) with *T. brucei* confirmed the central position of TNF in parasite control but also its detrimental involvement in pathology. The level of parasitemia was elevated in infected $TNF^{-/-}$ mice. In parallel the *T. brucei*-related immunopathology was attenuated [237]. An involvement of the closely related cytokine LTα could be excluded [238]. In a similar approach using a different TNF-deficient mouse strain on a different mixed background and the related parasite, *Trypanosoma congolense*, infected mice were shown to be highly susceptible and died sooner than control mice infected in parallel [239]. Finally, in comprehensive infection experiments using *T. brucei* to infect the strains C3H/HeN, BALB/c, C57BL/6, CBA/Ca, $TNFR1^{-/-}$ and $TNFR2^{-/-}$, a range of clinical parameters was tested [235]. A common parameter in these models was a correlation between the presence of soluble TNFR2 and the absence of trypanosomiasis-associated pathology [235]. However, the data presented on the outcome of infection of TNFR1 versus TNFR2-deficient mice didn't support a proposed protective role of TNFR2 because the immunopathology in TNFR2-deficient mice was decreased and the final outcome was unchanged.

Taken together, the complexity of the infection and the large part the immune system plays in the pathology of trypanosomiasis makes a clear assessment of the role of TNF difficult. However, the current observations make an involvement in both the protective immune response as well as the immunopathology highly likely.

Table 2.3 Contribution of genetic background to immunity to experimental trypanosomiasis in TNF family knockout mice.

Genetic Background / Treatment	***Trypanosoma* spp.**	**Clinical Outcome**	**Reference**
BALB/c + recombinant TNF	*T. cruzi*	Increased mortality	[232]
BALB/c + anti TNF mAb (TN3)	*T. cruzi*	Increased mortality/ Increased Cachexia	[234]
BALB/c + anti TNF mAb (1F3F3)	*T. cruzi*	Attenuation of Cachexia, improved protective immune response	[234]
C57BL/6-*Tnfrsf1a* (TNFR1) *(9 gen)*	*T. cruzi*	Increased Parasitemia, increased mortality	[236]
$TNF^{-/-}$ (B6x?) unknown number of backcrosses	*T. brucei AnTat1.1E*	Increased Parasitemia/ decreased morbidity	[237]
TNFR1 (C57BL/6 background)	*T. brucei AnTat1.1E*	Strong anaemia and weight loss. Fatal outcome	[238]
TNFR2 (C57BL/6 background)	*T. brucei AnTat1.1E*	Decreased anaemia and decreased weight loss. Fatal outcome	[238]
$TNF^{-/-}$ (ES cells: C57BL/6 x CBA) five back-crosses to C57BL/1 = F5; F5 x IL1R IC F5 hemizygous IC with C57Bl/6 + A/J	*T. congolense*	Increased mortality	[239]

mAb, monoclonal antibody, TNFR, TNF receptor; ES, embryonic stem cells.

TNF in experimental cutaneous leishmaniasis

The intracellular protozoan parasite *Leishmania* spp. is an important human pathogen. It infects significant numbers of people in the developing world and increasing numbers in the developed world. Aproxametely 12 million people are estimated to be infected worldwide with cutaneous leishmaniasis with an estimated two million new infection reported every year[81, 82, 85]. Infection with *Leishmania* spp. results naturally from transmission of metacyclic promastigotes by the sand fly hosts *Phlebotomus* spp. and *Lutzomyia* spp. during a blood meal [240, 241] and can cause a spectrum of diseases ranging from the relatively benign cutaneous leishmaniasis, which presents as a skin lesion that eventually resolves, to diffuse cutaneous leishmaniasis, a sub-form that occurs in immuno-compromised patients, muco-cutaneous leishmaniasis, which infects the mucosa and can be extremely disfiguring and, finally, visceral leishmaniasis, which is fatal if left untreated[82].

Leishmaniasis, as a model, has been used to analyse T cell activation, to investigate differential effector strategies in response to complex antigens and has been instrumental in establishing the classification of Th1 and Th2 cells, which is well-defined in inbred mouse strains [63, 68]. Resolution of an established infection is mediated by IFN-γ produced by $CD4^+$ T cells in resistant mouse strains such as C57BL/6, the hallmark of a Th1 response [242]. In contrast, a susceptible strain, the BALB/c mouse, develops a polarized IL-4 and IL-10-mediated $CD4^+$ T cell response, which is characteristic of a Th2 response. The IFN-γ response allows the infected host cells, mostly macrophages [243], to develop strong cellular leishmanicidal activities including expression of iNOS and consequently, the production of nitric oxide [244].

The role of TNF in the development of a protective immune response to *Leishmania major* infection has been analysed extensively in experimental cutaneous leishmaniasis (Table 4), yet this analysis has yielded contradictory results. Treatment with recombinant TNF resulted in reduced lesion size and parasitic burden [107, 245] whereas the application of neutralizing anti-TNF antibodies caused a transient aggravation of the symptoms [106, 107, 246, 247]. This approach of an inoculation of a *L. major*-infected mouse with a neutralizing agent was perfected in a transgenic model that continuously produced a TNFR1-Ig fusion protein. These mice developed serious non-healing lesions at the site of infection [108]. Taken together, the sometimes contradictory conclusions based on these experimental approaches suggested TNF as a co-factor acting in concert with other cytokines. Two recent developments have altered this point of view. First, in some rheumatoid arthritis sufferers treated with TNF antagonists, the effects of TNF neutralisation during leishmaniasis could be observed for the first time in humans. In a few published cases the consequence of blocking TNF was a recurrence of the clinical symptoms of leishmaniasis [119, 248], supporting the notion of an important role for TNF.

Second, a range of infection experiments has been published utilising gene-targeted mice deficient for TNF, memTNF, TNFR1 and TNFR2 (Table 2.4). TNFR1 and TNFR2 gene-deficient mice (on a mixed C57BL/6 x 129 background) developed a protective Th1-type response and expressed IFNγ and iNOS [109, 110]. In contrast, the outcomes of *L. major* infection in two C57BL/6 strains deficient for both soluble and membrane TNF [5] or deficient only for soluble TNF [19] (established directly on a C57BL/6 background) were unexpected.

The sole presence of membrane-bound TNF allowed for a protective immune response and

Table 2.4 Contribution of genetic background to immunity to cutaneous leishmaniasis in TNF family knockout mice.

Genetic Background/ Treatment	***Leishmania* spp.**	**Clinical Outcome**	**Reference**
C57BL/6	*L. major*	Resistant	[68]
TNF antagonists (including transgenic TNFR1)	*L. major*	Transient aggravation of symptoms	[107, 108, 247]
Recombinant TNF	*L. major*	Reduced lesion size, reduced parasite burden, attenuation of symptoms	[107, 245]
C57BL/6-*Tnf*	*L. major*	Susceptible	[112]
C57BL/6-*Tnfrsf1a* (TNFR1*) (8 gen)*	*L. major*	Resistant	[109]
C57BL/6-*Tnfrsf1b* (TNFR2*) (B6 x 129 random background)*	*L. major*	Resistant	[110]
TNFR1/2 *(B6 x 129 random background)*	*L. major*	Resistant	[110]
C57BL/6-memTnf$^{\Delta/\Delta}$	*L. major*	Resistant	[113]
C57BL/6-*Ltb*	*L. major*	Susceptible (Protective immune response disturbed by lack of LN)	[249]

TNFR, TNF receptor; *Ltb*, gene for lymphotoxin beta; LN, lymph nodes.

was sufficient to resolve the lesion after infection [113], while C57BL/6-*Tnf* mice succumbed rapidly to infection [112]. This clearly points to a central role for soluble TNF in the defence against *L. major*, which seems to be masked by genetic factors in the original infection experiments. Interestingly, a further reason for the obvious discrepancy between the various published disease courses of TNF- and TNFR-deficient mice infected with *L. major* can at least in part be explained by the different strains used for the infections. As published recently, different *L. major* isolates result in significantly different clinical outcomes in the C57BL/6-*Tnf* strain [114]. The reason behind the fatal outcome in

L.major-infected C57BL/6-*Tnf* still remains puzzling. The TNF-deficient mouse strains mount strong, cell-mediated IFN-γ responses to *L. major*, highlighting the obvious need for IFN-γ independent pathways to facilitate leishmanicidal activity. The argument for a central role of TNF in the anti-*Leishmania* immune response has been strengthened by observations in the *Leishmania donovani* model of visceral leishmaniasis. After blocking of TNF, infected mice were unable to acquire resistance and resolve the infection [250].

Concluding remarks

The role of TNF has been investigated in many experimental models of infection. The results which have been reported are not always consistent and sometimes surprisingly contradictory. This review highlights that the genomic make-up of knockout models used to study TNF or TNFR could be a contributing factor to the different outcomes in experimental models of intracellular infection, keeping in mind that back-crossing five generations leaves approximately 3% of non-linked genes from the founder ES cell line.

With regard to the location of TNF in the genome and its proximity to immunologically relevant genes, this becomes especially important to correctly interpret the contribution of TNF to immunity and pathology in response to parasitic challenge. Furthermore, the four highlighted examples, malaria, toxoplasmosis, trypanosomiasis and leishmaniasis, indicate that protective mechanisms involving the TNF/TNFR axis which deal with different intracellular parasites are not canonical but vary in response to the pathogen. Thus consideration for both the protective role but sometimes also the contribution to pathology afforded by TNF/TNFR, need to be considered in future therapeutic strategies aimed at targeting TNF. An essential prerequisite to this is an improvement of our still rudimentary knowledge of the host response and the protective mechanisms that are at work during parasitic infection.

Chapter 3: Fatal leishmaniasis despite a strong Th-1 type response.

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Introduction

Cutaneous leishmaniasis is a disease endemic in the tropics and subtropics and is caused by a range of different protozoan parasite species such as *L. major* [82, 251]. The cutaneous infection is transferred by the bite of a sandfly [241] and the clinical manifestation is normally limited to superficial skin lesion that heals without further treatment but can show an aggravated course with widespread tissue destruction [82]. The immune response to this infection within skin and periphery has been analysed extensively [252, 253] but is complex and still not entirely understood. The predominant hypothesis is based on considerable evidence from mouse experiments and states that ultimately, protection is based on an early presence of IL-12 [254] and an initial boost by IFNα/β [255]. The extent and consequences of these events (mainly the production of IFN-γ), depend on the genetic background of the infected mouse. Infected macrophages start to express large amounts of the enzyme iNOS and to produce NO [256] following activation by IFN-γ. This effector molecule allows macrophages to kill the pathogens they host in their phagolysosomes and its presence has correlates directly with resistance to *L. major* infection [244]. Other effector molecules such as NADPH oxidase (*gp91-phox homologue*) are of secondary relevance for survival, but are important for the elimination of the parasite from the host [257]. In C57BL/6 mice, the lesion resolves within approximately 8 weeks. In contrast, the immune response to *L. major* in the genetically susceptible BALB/c mice shows an early IL-4 peak, resulting in a progressive infection and ultimately, death. This dichotomy of the cytokine responses was used in large part to develop the Th1-Th2 paradigm, which interprets the difference of the cytokine expression as causal for the different clinical outcomes. The simplicity and validity of this paradigm has been questioned by recent observations in mouse strains congenic for two resistance loci which

display a dissociation of classical Th1/Th2 dichotomy and resistance to *L. major* [258-262]. Of note in this context, B6.TNF$^{-/-}$ mice cannot control the *L. major* infection despite producing IFN-γ. The *L. major*-specific T cells of B6.TNF$^{-/-}$ mice displayed strong IFN-γ expression *in vitro* [112], but *in vivo* they could not control and confine the infection, which spread to visceral tissues and ultimately killed the animals [112]. Since the presence of IFN-γ has been assumed to be indispensable for the control of *L. major* [263], and since the expression of sufficient amounts of IFN-γ was thought to be TNF-dependent [264], these results from B6.TNF$^{-/-}$ mice are somewhat counterintuitive.

To address this issue, I infected all relevant TNF and TNFR (TNFR1 and TNFR2) deficient mouse strains that had been backcrossed to or established on the C57BL/6 genetic background and compared the course of disease, the presence of peripheral IFN-γ and, NO, *in vitro* and *in vivo*.

Materials and Methods

Mice

The gene-targeted C57BL/6 mouse strain deficient for soluble and membrane TNF (B6.TNF$^{-/-}$) or for soluble TNF (B6.memTNF$^{\Delta/\Delta}$) only was generated on a genetically pure C57BL/6 (B6.WT) background as described [5, 19]. The B6.TNFR1$^{-/-}$ (Jackson stock number: 003242) and B6.TNFR2$^{-/-}$ mice (Jackson stock number: 002620) were obtained from Jackson Laboratories and had been backcrossed more than 10 times or had been established on a C57BL/6 background, respectively [265]. The screening procedure followed the protocols published previously [5, 265]. All animals were kept under specific pathogen free conditions at the Animal Research Facilities of the Comparative Genomics Centre at James Cook University or the Queensland Institute of Medical Research. All experiments followed protocols approved by the animal ethics committee of JCU, Townsville, the Queensland Institute of Medical Research (QIMR), Brisbane, or the Government of Mittlelfranken, Ansbach, Germany. Mice of 8-12 weeks of age were used in all experiments.

Parasites and Infection

The virulent *L. major* isolate MHOM/IL/81/FE/BNI was maintained through serial passage in BALB/c mice *in vivo* and cultured *in vitro* in Novy-Nicolle-MacNeal blood agar slants in RPMI containing 10% new born calf serum, Penicillin/Streptomycin, Non Essential Amino acids and 10mM HEPES [266], all supplied by Invitrogen. For infection,

stationary phase *L. major* promastigotes were used between passage two and six and $3x10^6$ parasites were injected in a volume of 40µl into one hind footpad. The infection site was monitored daily and the increase in lesion size was noted twice weekly by measuring the footpad thickness with a metric caliper (Kroeplin Schnelltaster, Schluechtern, Germany). The increase in footpad thickness (percentage) was determined by the formula: Thickness of infected footpad/mean thickness of non-infected footpad x 100. Parasite burden was calculated as a proportion of tissue weight at day 28 after infection using a limiting dilution method and L-Calc software version 1.1 (Stem Cell Technologies; www.Stemcell.com), which performs a generalized Pearson Chi-squared test [112].

Culture and infection of macrophages

Bone marrow (BM) cells were harvested from the femurs and tibias of B6.WT or gene-deficient mice using a 26g needle and red blood cells lysed in 0.17 M sterile ammonium chloride and 20 mM HEPES buffer for 10 minutes at 4°C. Cells ($1x10^5$ cells/ml) were seeded into Teflon bags containing 50mL RPMI-1680 supplemented with 10% fetal bovine serum, 5% horse serum, L-glutamine, non-essential amino acids and sodium pyruvate (Invitrogen) in the absence of antibiotics, as published [267]. L929 conditioned medium (a gift from Dr. Matt Sweet, University of Queensland, Brisbane) was added as a source of macrophage colony stimulating factor (M-CSF) to a final concentration of 10% - 15% after titration on primary BM cells. Cells were incubated for 7-10 days at 37°C at 5% CO_2.

For cytokine assays, macrophages ($1x10^6$cells/ml) were seeded into 8 or 16 well chamber

slides or into flat bottom 96 well plates (Nunc, Invitro Technologies). After two hours, non-adhered cells were washed off and IFN-γ (20ng/ml, Invitrogen) was added for a period of three hours prior to infection with stationary phase *L. major* promastigotes at a multiplicity of infection (MOI) of three. Media was harvested at 16 hours and extracellular promastigotes removed by extensive washing with PBS. Cells were then fixed and stained in Quik Diff (Thermo-Shandon). For kinetic analysis, superanatants were harvested at 16, 40 and 70 hour time points; media, and cytokines were exchanged at both 16 and 40-hour time points

Flow Cytometry

Tissue of draining popliteal LN or footpad lesions was incubated with Collegenase D (1mg/ml, Roche, Brisbane, Australia) and DNase 1 (100U/ml, Sigma-Aldrich, Sydney, Australia), for 30 minutes at 37°C, disrupted by mechanical disruption between frosted glass slides and single cell suspensions prepared. Cells were filtered through 60μm nylon mesh or 40μm cell strainers (BD Biosciences) to remove tissue debris. Prior to FACS staining the cells were blocked with anti-CD16/32 (clone 2.4G2, eBioscience, San Diego, USA) or 10% rat serum (IMVS, Adelaide, Australia). Cells were stained with rat anti-mouse antibodies against B220 (RA3-6B2, Pacific Blue or APC-Cy7), CD4 (RM4-5, PerCP-Cy5.5 or Pacific Blue), IFN-γ (XMG1.2, Alexa Fluor-488, Armenian-hamster anti-mouse TCR beta-chain (H57-597, APC or biotin/Streptavidin Pacific Orange), CD3 (145-2C11, PE-Cy7). Multicolour staining of single cells for surface antigens was performed essentially as published [112]. Data were accquired either using a Cyan ADP (Beckman Coulter, Fullerton, CA) or an Aria II (BD Biosciences). Analyses were performed using

FloJo version 8.86 (Tree Star Inc.).

Cytokine Analysis.

T cells were isolated from *L. major* infected pLN. Intracellular cytokine staining was performed on antigen re-stimulated $CD4^+$ T cells. The cells were re-stimulated using freeze-thawed *L. major* antigen [95] (MOI equivalent = three) for 72 hours. For the last six hours of culture, the cells were in the presence of PMA/ionomycin (Sigma-Aldritch) and Golgi-Stop (BD Biosciecnes). Subsequently, the cells were stained for surface antigens, fixed, permeabilised using FoxP3-FixPerm buffer (Biolegend, Perth, Australia) and stained for IFN-γ, IL-14 and IL-17 following the manufacturer's instructions.

Serum was collected from experimental animals weekly during the course of infection. Cell culture supernatants were collected from *L. major* infected BM macrophages at various time points. Reactive NO, as measured by nitrite (NO_3^-,), was determined in tissue culture supernatant by adding equal parts freshly prepared Griess reagent containing 1:1 of (1% Sulphanilimide (Sigma-Aldrich) in 5% Phosphoric acid and 0.1% N- (1-naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich) in triplicate using sodium nitrite as a standard. The mixture was incubated for 10 minutes prior to reading on a Versa MAX microplate reader (Molecular Devices, CA, USA) at 540nm [112]. Cytokine levels were measured in mouse serum and tissue culture supernatants using mouse inflammation cytokine bead array (CBA, BD Biosciences). CBAs were acquired using an ARIA II and FCS 2.0 files were analyzed using FCAP Array (Soft Flow Inc, Minnesota, USA)

Two-step Real Time PCR was performed on total RNA extracted on footpad lesions using

Table 3.1 Real Time PCR primers.

β-actin: fw	AAT CCT GTG GCA TCC ATG AAA C
β-actin: rv	CGC AGC TCA GTA ACA GTC CG
Gata3: fw	GAG GTG GAC GTA CTT TTT AAC AT
Gata3: rv	GGC ATA CCT GGC TCC CGT
Hprt: fw	GTT GGT TAC AGG CCA GAC TTT GTT G
H*prt*: rv	GAG GGT AGG CTG GCC TAT AGG CT
Il-10: fw	GGT TGC CAA GCC TTA TCG GA
Il-10: rv	ACC TGC TCC ACT GCC TTG CT
Il-17A: fw	TCT GTG TCT CTG ATG CTG TTT GC
Il17A: rv	ACG GTT GAG GTA GTC TGA GGG C
Ifn-γ: fw	AGA GCC AGA TTA TCT CTT TCT AC
Ifn-γ: rv	CTT TTT TCG CCT TGC TGC TG
Nos2: fw	TGC CCC TTC AAT GGT TGG TA
Nos2: rv	ACT GGA GGG ACC AGC CAA AT
Rorγt: fw	CCG CTG AGA GGG CTT CAC
Rorγt: rv	TGC AGG AGT AGG CCA CAT TAC A
Tbx21: fw	CAA CAA CCC CTT TGC CAA AG
Tbx21: rv	TCC CCAA GCA AGT TGA CAGT

Trizol (Invitrogen) or from $1x10^6$ FACS purified $CD4^+$ naïve ($CD62L^+$ $CD44^-$) or $CD4^+$ activated ($CD62L^-$ $CD44^+$) splenic T cells from mice infected with *L. major* mice at day 50. Cells were lysed using Trizol (Invitrogen) followed by RNA isolation using Purelink RNA micro kit (Invitrogen), as per the manufacturers instructions. Synthesis of cDNA was performed using Supercript III (Invitrogen) following DNase treatment of RNA using RQ1 RNase free DNase (Promega, Sydney, Australia). Gene expression was performed using SYBR-green-ER qPCR kit (Invitrogen) or Brilliant II SYBR-green (Agilent Technologies, Integrated Sciences, Willoughby, NSW) and run on a Corbett Rotor Gene 6000 (Qiagen, Doncaster, Australia) and analyzed using REST 2009 gene expression

software (Qiagen) to determine relative expression of genes. PCR primers were designed using Vector NTI (Invitrogen) (See Table 3.1)

Immunofluorescence Microscopy

Draining popliteal LNs were dissected and rapidly frozen in Tissue Tek optimal cutting temperature media (OCT) (ProSciTech, Townsville, Australia) in liquid nitrogen vapor and stored at -80°C. Sections of 10μm were cut using a cryotome (ThermoShandon), air-dried and fixed in acetone at -20°C. Prior to staining, sections were re-hydrated in PBS/1% BSA for 60 minutes followed by FcR blocking with anti CD16/CD32 (BD Biosciences). Polyclonal IgG antibodies against *L. major* (clone; V121, MHOM/IL/67/Jericho II) were purified from rabbit serum (a kind gift from Dr. Emanuela Handman, Walter and Eliza Hall Institute, Melbourne) using Protein G-Sepharose 4B (Invitrogen), followed by labeling with Cy5 mono-reactive dye (Amersham Biosciences, Buckinghamshire, England) as described by the manufacturers.

Staining of pLN sections was performed using polyclonal rabbit anti *L. major* Cy5, mouse anti-mouse iNOS-FITC (6/iNOS/NOS type II; BD Biosciences), and rat anti mouse B220 biotin (RA3-6B2; BD Biosciences). Secondary staining was performed using Streptavidin-Alexa 546 (Invitrogen) before being mounted with Mowiol (Calbiochem, La Jolla, CA) containing 2.5% DABCO (Sigma-Aldrich,) to prevent fading and allowed to dry overnight. Sequential images were acquired using a Zeiss LSM710 Confocal microscope in channel mode.

Statistics

Statistical analysis was performed using a non-parametric Mann Whitney test or a Kruskall-Wallace with Dunn's multiple comparison test or two-way ANOVA with Bonferroni correction to test for multiple hypotheses. Mean or median values ± SE or SEM are shown as indicated. Analysis was performed using GraphPad Prism 5.0b for Macintosh (GraphPad Software, San Diego California USA, www.graphpad.com). Each experimental group was compared to B6.WT controls. Significance values of $p<0.05$ were considered to be significant with * $p<0.05$ and ** $p<0.01$ respectively.

Results

Clinical progression of experimental cutaneous leishmaniasis in the absence of TNF or its receptors

The published clinical outcomes of *L. major* infection in TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice [109, 110] are significantly different from the infection of TNF$^{-/-}$ mice [112]. These discrepancies have been attributed to some extent to the use of different *L. major* isolates [114]. The impact of the genetic heterogeneity of TNF/TNFR knockout strains on clinical outcome, as well as the TNF-dependent leishmanicidal effector mechanisms, have not yet been analysed comprehensively. Therefore, I infected B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$, B6.TNFR2$^{-/-}$ and B6.memTNF$^{\Delta/\Delta}$ strains [5, 19, 265] with the virulent *L. major* isolate BNI and monitored the course of disease (Figure 3.1). The different outcomes (lesion size) were statistically compared to infected C57BL/6 controls. We were not able to detect a significant difference between the infected memTNF$^{\Delta/\Delta}$ mice and the B6.WT control mice, in accordance with previous results [113]. In contrast, *L. major*-infected B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$ and B6.TNFR2$^{-/-}$ mice all showed chronic lesion development different from the B6.WT control mice ($p<0.001$) from day 21 (B6.TNF$^{-/-}$, B6.TNFR2$^{-/-}$) and day 28 (B6.TNFR1$^{-/-}$) onwards. There was however, no statistical difference between either B6.TNFR1$^{-/-}$ or B6.TNFR2$^{-/-}$ and B6.TNF$^{-/-}$ with the exception of day 28 where B6.TNFR1$^{-/-}$ displayed a smaller overall lesion and day 42 where B6.TNFR2$^{-/-}$ mice showed a brief significant improvement. At day 56, all control mice had survived. The infected TNF- and TNFR1-deficient mice had to be terminated in accordance with animal ethics because the animals developed signs of distress. *L. major*-infected TNFR2-deficient mice developed large lesions, which were comparable to TNF and TNFR1 mice, but

ultimately, survived the infection.

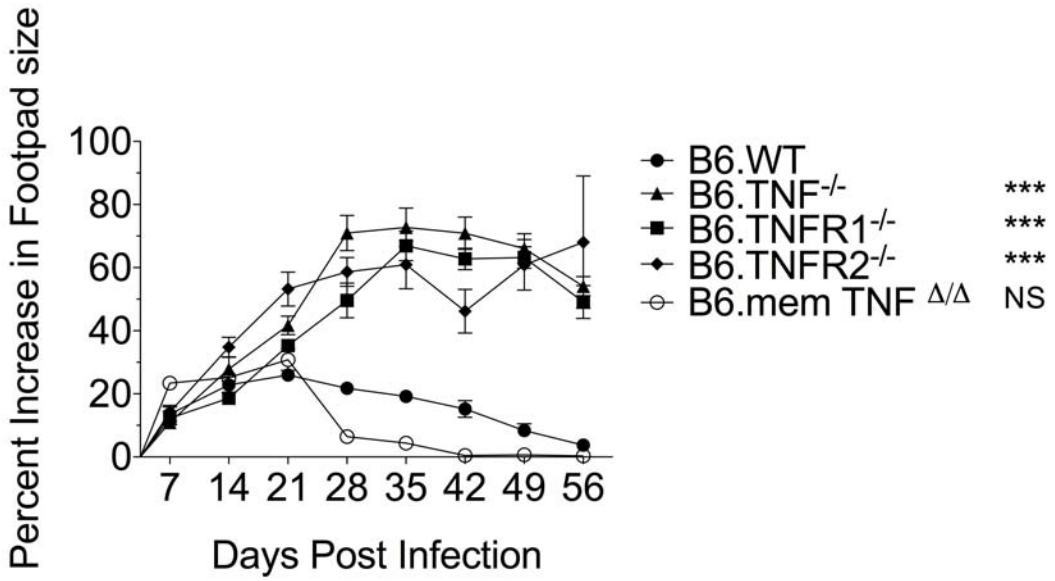

Figure 3.1 Course of infection in TNF and TNFR deficient mouse strains:
The lesion sizes of B6.TNF$^{-/-}$ (n=34), B6.memTNF$^{\Delta/\Delta}$ (n=5), B6.TNFR1$^{-/-}$ (n=27) and B6.TNFR2$^{-/-}$ (n=27) mice, which had been infected subcutaneously with *L. major* in one hind footpad were determined and compared to B6.WT (n=36). The data are presented as percent increase in footpad lesion size. The combined results of four experiments are shown as mean (± SEM) and the number of animals stated above is the maximal number at the beginning of the experiment. All mice were either established on a C57BL/6 background or had been backcrossed more than 10 times (TNFR1$^{-/-}$), respectively. Furthermore, the infections were carried out using the *L. major* isolate MHOM/IL/81/FE/BNI. Therefore, the differences in clinical outcome were combined for analysis using a two-way ANOVA with Bonferroni correction to test for multiple hypotheses. *** P<0.001, NS- Not significant.

Induction of IFN-γ response in B6.TNF$^{-/-}$ mice during L. major infection.

To determine how the absence of TNF impacted on the development of the adaptive immune response C57BL/6, B6.TNF$^{-/-}$, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ were infected with 3x10^6 *L. major* promastigotes in the posterior footpad. Infected tissue from the footpad, draining pLNs and serum were collected at days 7, 14, 21, 28, 35 and 48 for real time PCR, intracellular cytokine staining and a cytometric bead array (CBA) analysis to determine the expression of a number of pro- and anti-inflammatory cytokines. Interestingly, in the serum of B6.TNF$^{-/-}$ or B6.TNFR1$^{-/-}$ mice, significantly higher concentrations of IFN-γ

were detected throughout the course of infection (Figure 3.2A). The expression level of IFN-γ mRNA in the footpad lesion was initially comparable between infected B6.WT and B6.TNF$^{-/-}$ mice, but was significantly elevated in the latter strain after day 35 of infection (Figure 3.2B). The expression of IL-4 was also analysed together with IL-17 at day 7 and 21. While IL-4 was present early in the infection in both B6.WT and B6.TNF-deficient mice, it failed to be maintained throughout the immune response, consistent with earlier publications [90, 92], while, IL-17 was virtually undetectable at any time point.

Expression analysis of transcription factors and cytokines in activated CD4$^+$ T cells in L. major infected B6.WT and B6.TNF$^{-/-}$ mice

The polarisation of T cell responses towards IFN-γ production is strongly regulated by the balance of a number of transcription factors including *Tbet* (*Tbx21*) and *Gata3* that regulate the expression of IFN-γ and IL-4, respectively [64-66]. To ascertain if the increased presence of IFN-γ observed in the absence of TNF during the course of experimental cutaneous leishmaniasis (Figure 3.2) resulted from an alteration in the expression of these transcription factors, the levels of transcription factors involved in T cell differentiation was examined by real-time PCR. Comparison of *Tbx21* expression of purified, activated CD4$^+$ T cells (CD62L$^-$ CD44$^+$) with naïve CD4$^+$ T cells (CD62L$^+$ CD44$^-$) from B6.WT and B6.TNF$^{-/-}$ mice at day 50 after infection showed an expected up-regulation in activated CD4$^+$ T cells by a median factor of 134 (111.43-170.87) and 34 (23.7-41.56) fold, respectively (Figure 3.3A). This increase in *Tbx21* expression in both genotypes was correlated with an observed increase in *Ifn-γ* expression (497-fold up-

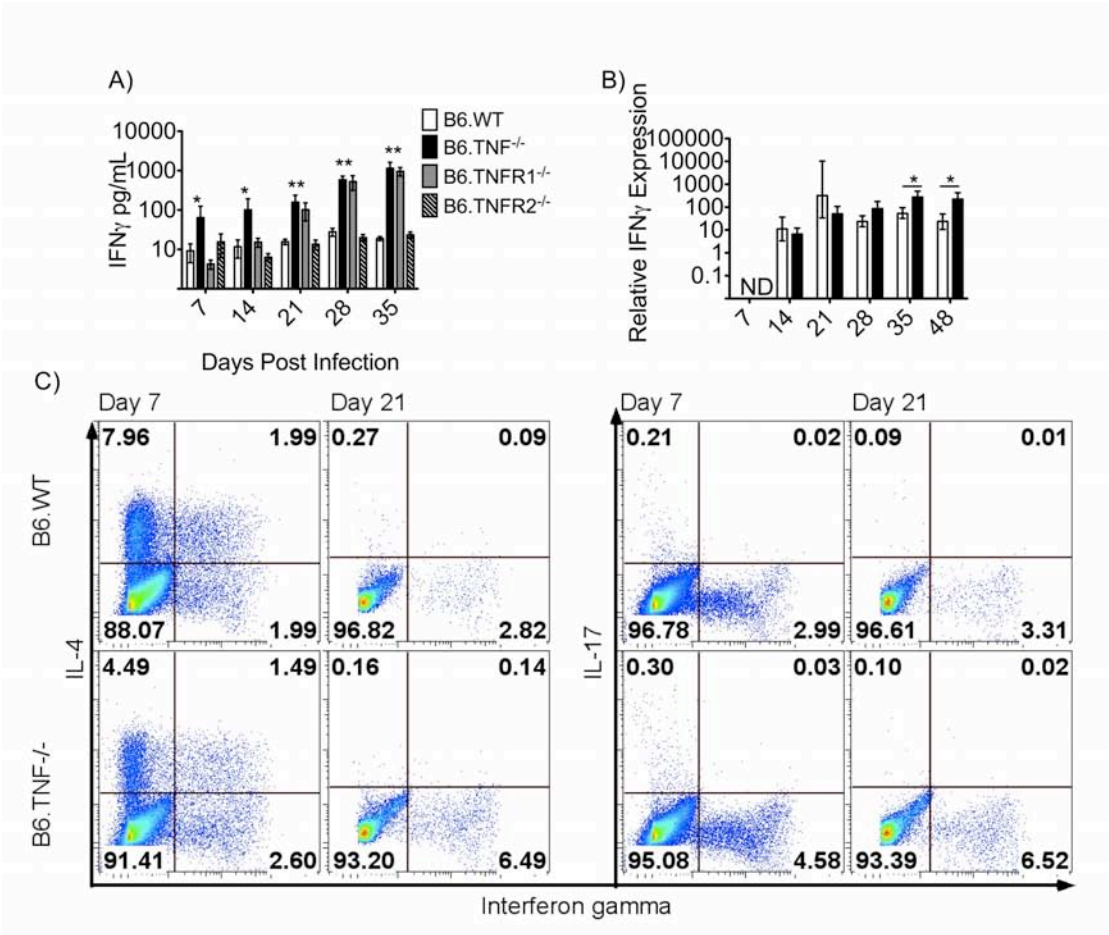

Figure 3.2 Infection of B6.TNF$^{-/-}$ mice with *L. major* results in elevated IFN-γ expression in serum, the infected tissue and T cells isolated from the draining LN.
IFN-γ concentrations were determined in serum of *L. major* infected wildtype or B6.TNF$^{-/-}$ mice over the course of disease (A). The data are presented as mean (± SEM; n=5-6 per genotype). Results are representative of at least 3 independent experiments. The non-parametric Mann Whitney U-test was used to test for statistical differences (* = $p<0.05$ ** = $p<0.01$). The relative expression of IFN-γ (B) in the footpad lesion of infected WT or TNF$^{-/-}$ mice was compared to uninfected controls. Relative expression was calculated relative to β-actin as described [268] using REST 2009 relative gene expression software (Qiagen). The data are presented as median (± SE; n= 3-5 mice, representative of 2 independent experiments). Cytokine expression in CD4^{+} T cells from draining LN of *L. major* infected mice was analysed (C). Intracellular flow cytometry was used to determine the expression of IFN-γ , IL-4 and IL-17 in CD4^{+} T cells at day 7 and 21 post infection. The experiment was performed twice.

regulation in B6.WT mice and 324-fold up-regulation in B6.TNF$^{-/-}$ mice), which is consistent with Th1 cell differentiation [64, 65] associated with resistance to *L. major* infection. The expression of *Gata3*, which regulates both IL-4 and IL-10 production [66, 269], was virtually unchanged by activation at this time point and was slightly reduced in B6.TNF$^{-/-}$ CD4^{+} T cells, as compared to B6.WT CD4^{+} T cells (1.6-fold up-regulation change in B6.WT compared to 0.7-fold down-regulation in B6.TNF$^{-/-}$ CD4^{+} T cells). Additionally, we analysed the expression of the transcription factor *Rorγt (Rorc)* which is characteristic of the pro-inflammatory Th17 T cell subset [69]. At this late stage of infection, it was up-regulated in both B6.WT (67.9-fold) and B6.TNF$^{-/-}$ (48.6-fold) CD4^{+} T cells to a similar extent, although expression of IL-17A was barely detectable either by real-time PCR (Figure 3.3B) or by intracellular cytokine staining (Figure 3.2 D and Figure 3.3 A and B).

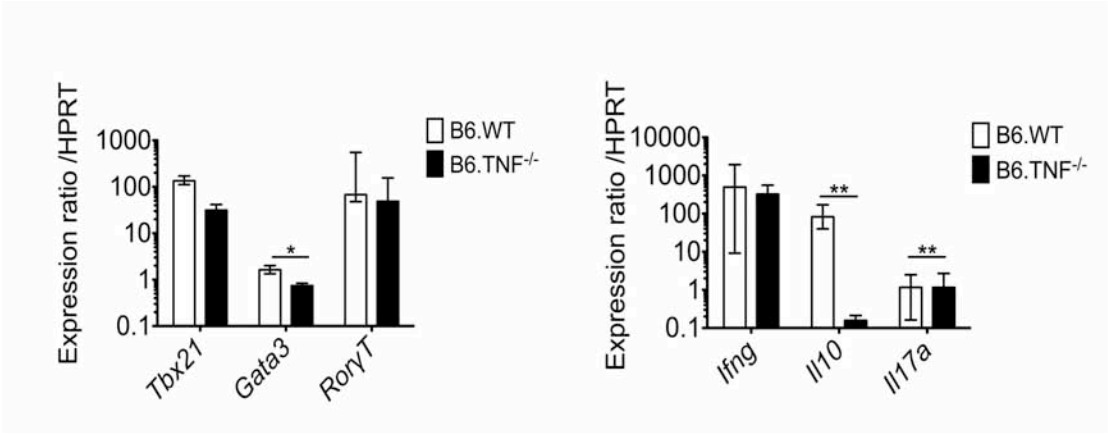

Figure 3.3 Expression of *Tbx21* is unchanged while expression of *Gata3* is reduced in activated TNF-deficient CD4^{+} T cells.
Naïve (CD6262L^{+} CD44^{-}) and activated (CD62L^{-} CD44^{+}) CD4^{+} T cells from B6.WT or B6.TNF$^{-/-}$ mice at day 50 after *L. major* infection were isolated from the spleens of individual mice. Relative expression analysis of transcription factors (A) and cytokines (B) was performed using 2 step real time PCR normalised to hypoxanthine guanine phosphoribosyl transferase (HPRT). In all cases gene expression in activated CD4^{+} T cells was compared to naïve CD4^{+} T cells from the same individual using REST 2009 relative gene expression software. Data are presented as median (± SE; n= 5-6 mice, one of 2 independent experiments is shown).

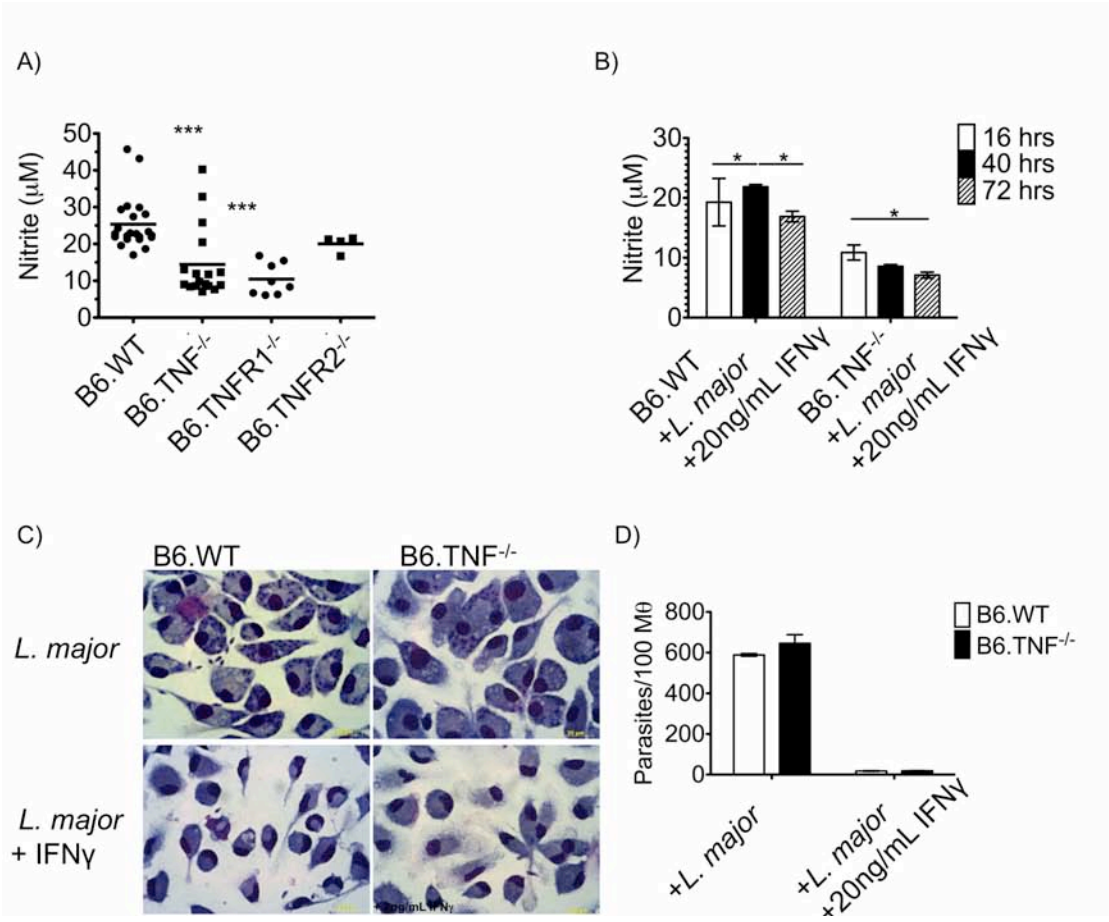

Figure 3.4 TNF- and TNFR-deficient macrophages exhibit decreased nitrite production but maintain leishmanicidal capacity in the absence of TNF *in vitro*.
A) BM-derived macrophages deficient in TNF or either TNFR1 or TNFR2 show decreased nitrite production following stimulation with 20ng/mL IFN-γ for 40 hours compared to wild type macrophages. In all cases nitrite production from unstimulated controls was below the detectable limit (data not shown). Results shown are combined from up to four experiments. Each experiment is represented as a single dot. (B6.TNFR2$^{-/-}$, n=4; B6.TNFR1$^{-/-}$: n = 8, B6.TNF$^{-/-}$: n = 17; B6.WT: n = 22, *** = p≤ 0.001). B) Kinetic analysis of IFN-γ stimulated BMM nitrite production following infection with *L. major* (MOI=3). C) Differences in leishmanicidal activity was determined by histological examination of infected macrophages grown on chamber slides after Quick-Diff staining. D) Quantification of leishmanicidal activity presented as number of amastigotes per 100 macrophages (±SEM).

Induction of iNOS does not correlate with anti leishmanicidal activity in B6.TNF$^{-/-}$ mice.

Nitric oxide is the major effector molecule responsible for life-long control of cutaneous leishmaniasisin mice [106, 244, 255]. Interestingly, the concentration of NO (Figure 3.4A), IL-10, IL-12 or MCP-1 *in vitro* (data not shown) did not directly correlate with the efficiency of leishmanicidal activity (Figure 3.4 C, D). Murine *L. major*-infected macrophages of all investigated genotypes, with the exception of B6.TNFR2$^{-/-}$ mice, produced reduced amounts of NO (approximately 50%, Figure 3.4 A), after stimulation with IFN-γ, and comparable levels of IL-10 and IL-12 (data not shown), yet they were still able to kill *L. major* parasites (Figure 3.4 C, D). To determine if the absence of TNF during infection with *L. major* impacted on the induction of iNOS *in vivo*, B6.WT and B6.TNF$^{-/-}$ mice were infected with *L. major* and footpads and pLNs were taken for real time PCR and immuno-fluorescence microscopy. In the footpad lesion, NO was readily detectable after day 14 post infection and there was no statistical difference in the induction of *Nos2* (*iNOS*) throughout the course of infection between B6.WT and B6.TNF$^{-/-}$ mice (Figure 3.5 A). Immunofluorescence at day 21 post infection, which correlated with the peak of acute disease in both B6.WT and B6.TNF$^{-/-}$ mice, highlighted that induction and localisation of iNOS is similar, with both B6.WT and B6.TNF$^{-/-}$ mice showing clustering of iNOS that was associated both dependently and independently with the presence of *L. major* amastigotes within the T cell zone of the draining pLN as detected by confocal microscopy (Figure 3.5B). Despite strong induction of iNOS in both the skin lesion and draining lymph nodes (Figure 5A and B), B6.TNF$^{-/-}$ mice show increased parasite burdens both in the footpad lesion ($p>0.05$) and, after visceralization of the parasites, in the spleen ($p<0.01$) (Figure 3.5C), and ultimately succumb to infection (see Figure 3.1).

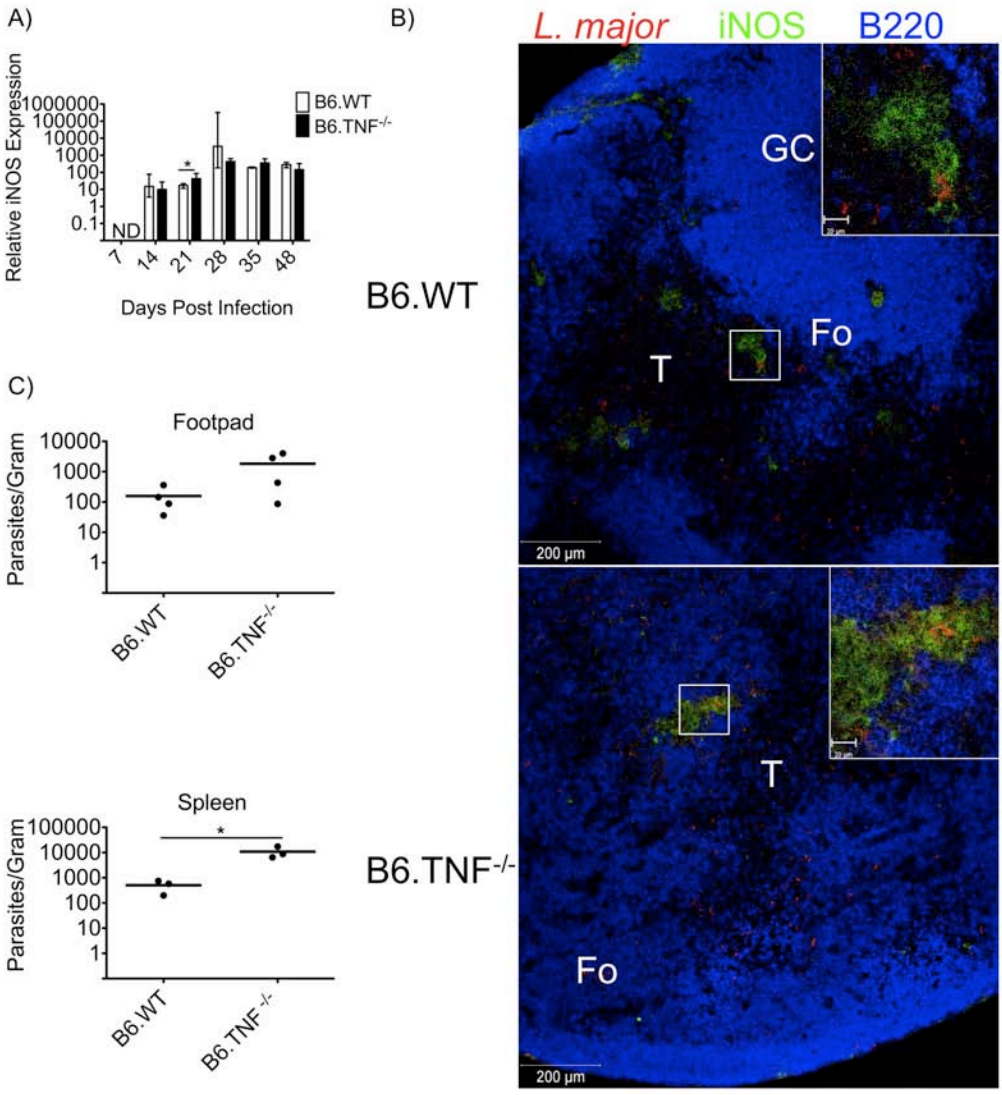

Figure 3.5 Induction of iNOS in B6.TNF$^{-/-}$ mice fails to control and contain *L. major* parasites within the draining LN *in vivo*.

A) The expression of iNOS was up-regulated in footpad lesions of B6.WT and B6.TNF$^{-/-}$ mice at similar kinetics during infection as measured by Real Time PCR using relative expression analysis compared to ß-actin expression with REST 2008 (Qiagen). The results are presented as median (± SEM; n=3). B) The spatial expression of iNOS (Green) was analysed at day 21 after infection (at the peak of acute disease in B6.WT mice) in conjunction with *L. major* parasites (red) and B220 (blue) in the draining pLN of B6.WT and B6.TNF mice. An overall merged image is shown at 10x magnification. Inset shows enlarged view of iNOS clustered regions identified in the image. The scale bar is equivalent to 200 µm in the overall image and 20 µm in the inset. Fo, B cell follicle; GC, Germinal Centre; T, T cell area. C) The parasite burden was determined in footpad and spleen of B6.WT and B6.TNF$^{-/-}$ mice at day 28 after infection by limiting dilution analysis in an endpoint assay and is shown as parasites per gram tissue using L-Calc software (n=4; * $p<0.01$; Mann Whitney U test).

Discussion

TNF and its receptors are essential in mediating resistance to infection by parasitic pathogens such as the obligate intracellular *L. major* [270]. In the absence of the pro-inflammatory cytokine TNF, the response to *L. major* is severely impaired resulting in a progressive infection, and eventually, a fatal outcome [112]. This is despite the induction of an IFN-γ-producing T cell response and the associated induction of innate effector mechanisms that are normally associated with protection [68, 271, 272].

While previous studies have implicated TNF as being essential to control infection with *L. major* [112], the individual role of TNFR1 and TNFR2 in leishmaniasis is less clear and is controversial in the context of the infection of B6.TNF$^{-/-}$ mice. In contrast to the rapidly fatal outcome of an infection of B6.TNF$^{-/-}$ mice with *L. major* [112], both TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice survived the infection but displayed different levels of resistance [110]. The TNFR1$^{-/-}$ mouse strain survived the infection and eliminated the parasites but did not resolve the skin lesion, while infection of TNFR2$^{-/-}$ mice showed that this receptor was not important for the immune response to *L. major*. An infection of a TNFR1/2 double knockout followed the TNFR1$^{-/-}$ phenotype [110]. In this study, I compared B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$ and B6.TNFR2$^{-/-}$ mice on identical genetic backgrounds using the *L. major* parasite isolate MHOM/IL/81/FE/BNI to account for differences caused by genetic variability of the infected mouse strain and the *L. major* isolate [114]. In my experiments, the previously observed differences could not be replicated. Surprisingly, both B6.TNFR1$^{-/-}$ and B6.TNFR2$^{-/-}$ mice displayed lesion development comparable to B6.TNF$^{-/-}$ mice suggesting overlapping roles for the two receptors in the pathogenesis of leishmaniasis. Interestingly, B6.TNFR2$^{-/-}$ mice displayed an increased lesion size but ultimately survived,

whereas both B6.TNF$^{-/-}$ and B6.TNFR1$^{-/-}$ mice succumbed to the infection. The lethal *L. major* infection in B6.TNF$^{-/-}$ mice [112] questions the previously published data in experimental cutaneous leishmaniasis using anti-TNF blocking agents [107] or TNFR-knockout strains, which invariably had non-fatal outcomes [110]. Further experiments will be necessary to reconcile these differences and to better comprehend the underlying biology of TNF in leishmaniasis.

Experimental cutaneous leishmaniasis in genetically inbred mice was the first model that showed a strain associated polarisation of IL-4 or IFN-γ production by CD4^{+} T cells, correlated in turn, with either disease susceptibility or resistance [68]. This observation was the basis of the Th1 and Th2 paradigm [63, 68]. The consequence of the blocking of IL-4 production for the development of susceptibility to *L. major* infection has been investigated extensively but some questions remain, especially in the light of new research [93]. Using anti-IL-4 (11B11) mAb induces a resistant phenotype with the corresponding induction of IFN-γ production. However, concurrent blocking of IFN-γ does not revert the resistance to susceptibility [94, 95]. Experimental cutaneous leishmaniasis in BALB/c mice in the absence of IL-4 or IL-4Rα resulted in complete or partial resistance, respectively, but did not result in a shift in the cellular immune response towards IFN-γ production commonly associated with protection [96]. The disparity observed between IL-4 or IL-4Rα-deficient mice could be attributed to the additional absence of IL-13 signalling in IL-4R$^{-/-}$ mice. These mice present with similar parasite burdens to BALB/c mice although different acute and chronic disease patterns have been noted [97, 98]. Interestingly, BALB/c IL-13-deficient mice (4 backcrosses) display a resistant phenotype following infection with *L. major* (LV39) [99]. Additional complexity is added when different *L. major* isolates are used for infection of IL-4- or IL-4Rα-deficient mice

resulting in a spectrum of clinical outcomes [97]. The mice differ in the control of lesion development, parasite numbers and parasite containment, ranging from a resistant phenotype to a fully susceptible state with necrotising lesions and parasite visceralisation [100].

For many years a major argument for a central role of IFN-γ in resistance to *L. major* infection has been that the polarising influence on $CD4^+$ T cell differentiation and the induction of essential effector mechanisms such as nitric oxide production in macrophages are critically dependant on IFN-γ [273-275]. This has been tested by administration of anti-IFN-γ mAbs to C3H/HeN mice,which prevents the development of natural resistance [90, 263]. Furthermore, resistant mice lacking either IFN-γ or IFN-γR fail to resolve *L. major* infection [276, 277]. However, several studies have called into question this fundamental role for IFN-γ in the sequence of events resulting in protection. The administration of anti-IL-4 mAb to susceptible BALB/c mice causes progressive uncontrolled infection of *L. major* [94] in parallel with an up-regulation of IFN-γ production. Concurrent neutralisation of this elevated IFN-γ did not change this resistance phenotype. However, mice congenic for known resistance loci (*Lmr1, Lmr2, Lmr3*) derived from either resistant C57BL/6 or susceptible BALB/c mice do not display the expected susceptible/resistant phenotype, despite expressing IL-4 or IFN-γ [151, 258, 260-262], suggesting that the IFN-γ / IL-4 cytokine profile alone is not a sufficient determinant of disease resistance. Resistant C57BL/6 mice carrying a BALB/c congenic region for *lmr1* (D17Mit57-D17Mit39) displayed increased susceptibility, while the reciprocal congenic strain (BALB/c containing C57BL/6 congene – D17Mit57-D17Mit129) trended towards an intermediate phenotype [261]. It is now becoming increasingly clear that the

exclusive focus on the balance between Th1 and Th2 cytokines, such as IFN-γ and IL-4, and their association with disease susceptibility has been too simplistic an explanation.

The synergistic relationship between IFNγ and other inflammatory agents such as TNF or LPS in mediating the sustained induction of the iNOS in macrophages (MΦ) through cooperative signalling through the transcription factors STAT1, IRF-1 and NFκB has been well described *in vitro* [273, 278-280] and *in vivo* [106]. The effect of autocrine or paracrine TNF on promoting synergy between NFκB and STAT1 signalling on iNOS transcription, however, appears to display a degree of redundancy. Stimulation with the Toll like receptor agonist LPS can overcome the block to NO production in both wild type BMM or thioglycolate elicited MΦ treated with anti-TNF mAb, as well as in B6.TNF$^{-/-}$ BMΦ [112]. While this synergy between IFNγ and TNF is clearly evident *in vitro* in regulating the production of nitric oxide by MΦ, there appears to be a level of redundancy *in vivo* as evidenced by expression of iNOS in the lesion and draining lymph node in B6.TNF$^{-/-}$. Interestingly, B6.TNF$^{-/-}$ and B6.TNFR1$^{-/-}$ mice, but not B6.TNFR2$^{-/-}$ mice produced IFNγ at levels in excess of that observed in resistant B6.WT mice and failed to resolve to their lesions. However, despite induction of iNOS, which is known to be involved in parasite clearance [244, 257, 281], B6.TNF$^{-/-}$ and B6.TNFR1$^{-/-}$ mice still failed to control parasite growth and dissemination.

The disassociation of IFN-γ from resistance to *L. major* infection in B6.TNF$^{-/-}$ and B6.TNFR1$^{-/-}$ mice, coupled with the maintained expression of iNOS in these animals, points to a central role for TNF:TNFR1 signalling in linking innate leishmanicidal effector mechanisms with the adaptive immune response. This would seem to occur upstream of intrinsic TNF signalling on innate immune cells and may point to an altered differentiation

pathway that renders the host refractory to IFN-γ, facilitating parasite growth and dissemination. Since TNF has been shown to not only contribute to monocyte /dendritic cell differentiation and activation in humans and mice [282], but also to the expansion of regulatory T cell networks [75], the absence of TNF:TNFR1 signalling may not result in one lethal immunological deficiency but could contribute to the lack of an efficient local immune response that is unable to prevent progressive infection. Furthermore, the sustained presence of large amounts of systemic IFN-γ throughout the course of leishmaniasis in TNF-deficient mice may be the consequence of ongoing infection and parasite dissemination. Instead of promoting protection by activating macrophages, overproduction of IFN-γ could result in immunopathology that contributes to a fatal outcome in response to infection with *L. major*.

Chapter 4: Modification of $CD4^+$ T cell responses to Leishmaniasis in the absence of TNF

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Running Title: Altered T cell responses in TNF-deficient mice during experimental cutaneous Leishmaniasis.

Introduction

TNF is produced early in the course of infection by a number of different cell types including macrophages [21], NK cells [23] and CD4 and CD8 T cells [63] and is involved in numerous aspects of both innate and adaptive immunity. The importance and complexity of this cytokine in regulating immunity to parasitic infection is apparent from studies using either neutralizing antibodies against TNF or with mice deficient for TNF [270]. Negation of TNF signalling in this manner can result in either beneficial or detrimental effects, ranging from attenuation of TNF mediated immuno-pathology, to exacerbated disease progression and failure to control parasite growth. This spectrum of TNF related effects can differ even within the same infectious agent depending on the genetic background of the host [270]. This has been exemplified in the context of infection with the protozoan parasite *L. major* in which mice deficient for TNF rapidly succumb to infection despite the production of a typically protective IFN-γ response [112].

The generation of T cell responses during cutaneous leishmaniasis is vital to controlling infection and has been well studied with respect to the genetic differences between susceptible and resistant strains of mice. This was accomplished primarily through studying a restricted *Leishmania* homologue of the mammalian receptor for activated C kinase (LACK) specific T cell population. [283-285]. Processing and selection of immuno-dominant LACK antigen by the non classical MHC II molecule DM (H2-DM) for presentation by H2 in mice having the $H2^D$ haplotype leads to an early expansion of IL-4 producing $V\alpha8^+$ $V\beta4^+$ $CD4^+$ T cells [284]. This antigen specific T cell repertoire is similarly expanded in resistant mice such as C57BL/6 and B10.D2 [283, 285] and under susceptibility promoting conditions in these resistant strains such as neutralisation of IFN-γ

at the time of infection also produced IL-4 [284, 286]. While IL-4 production by these LACK specific T cells is a hallmark of infection in susceptible mice it is not sufficient to promote the extreme cytokine polarisation commonly attributed to the Th2 phenotype observed [287]. To further complicate the situation it has recently been demonstrated that strain specific differences in the processing of LACK antigen occur in association with IL-4 production from $V\alpha8^+$ $V\beta4^+$ $CD4^+$ T cells from mice expressing either $H2^D$ recognizing LACK residues AA156-173 or $H2^B$ recognizing AA293-305, respectively [288]. That may provide a mechanism for the deviations in T cell responses between these susceptible and resistant strains.

TNF previously had been shown to act as a co-stimulatory molecule for T cells, playing an important role in regulating effector T cell function and cytokine responses [76, 289], but also in regulating the development of induced regulatory T cells [75]. Since the dynamics of the T cell response to infection with *L. major* is a strong determinant in the outcome of the infection, and the absence of TNF results in a fatal outcome, the contribution of TNF in facilitating T cell activation and function was explored in the context of active infection with *L. major* in mice deficient in aspects of TNF signalling on the same $H2^b$ background.

Materials and Methods

Mice

The gene-targeted C57BL/6 mouse strain deficient for soluble and membrane TNF (B6.TNF$^{-/-}$) or for soluble TNF (B6.memTNF$^{\Delta/\Delta}$) only was generated on a genetically pure C57BL/6 (B6.WT) background as described [5, 19]. The B6.TNFR1$^{-/-}$ (Jackson stock number: 003242) and B6.TNFR2$^{-/-}$ mice (Jackson stock number: 002620) were obtained from Jackson Laboratories and had been backcrossed more than 10 times or had been established on a C57BL/6 background, respectively [265]. The screening procedure followed the protocols published previously [5, 265]. All animals were kept under specific pathogen free conditions at the Animal Research Facilities of the Comparative Genomics Centre. All experiments followed protocols approved by the animal ethics committee of James Cook University, Townsville, Australia.

Parasites and Infection

The virulent *L. major* isolate MHOM/IL/81/FE/BNI [290] was maintained through serial passage in BALB/c mice *in vivo* and cultured *in vitro* in Novy-Nicolle-MacNeal blood agar slants as described previously [266]. For infection, stationary phase *L. major* promastigotes were used between passage 2 and 6 and $3x10^6$ parasites were injected in a volume of 40µl into one hind footpad.

Flow Cytometry

Single cell suspensions were prepared from spleens, draining pLN or footpads by incubating minced tissue with Collegenase D (1mg/ml, Roche and DNase 1 (100U/ml, Sigma-Aldrich), for 30 minutes at 37°C, disrupted by mechanical disruption between frosted glass slides. Cells were filtered through 60μm nylon mesh or 40μm cell strainers (BD Biosciences) to remove tissue debris. Prior to FACS staining the cells were blocked with anti CD16/32 (clone 2.4G2, eBioscience) where appropriate.

Cells were stained with rat anti-mouse antibodies against B220 (RA3-6B2, Pacific Blue or APC-Cy7), CD90.2 (53-2.1, APC), CD4 (RM4-5, PerCP-Cy5.5 or Pacific Blue), CD8 (53-6.7, Pacific Blue), CD25 (PC61, APC; 7D4, FITC), CD44 (IM7, PeCy7), CD62L (MEL-14, APC), CD45.1 (A20, PE), CD45.2 (104, FITC), GITR (DTA-1, PeCY7), IL-10 (JES5-16E3), IFN-γ (XMG1.2, PE), V beta 4 TCR (KT4, PE), V beta 5.1/5.2 TCR (MR9-4, FITC) and Armenian-hamster anti-mouse TCR beta-chain (H57-597, APC) or CD3 (145-2C11, PE-Cy7).

Multicolour staining of single cells for surface antigens was performed essentially as published [112]. Determination of viability was performed using propidium iodide (Invitrogen) exclusion. Regulatory T cells were identified by intracellular staining using FoxP3-FixPerm buffer (Biolegend) and the use of anti mouse FoxP3 (FJK-16a, APC) which was purchased from, eBioscience). Data were acquired either using a Cyan ADP (Beckman Coulter, Fullerton, CA), an Aria II (BD Biosciences) or an LSR Fortessa (BD Biosciences). Analyses was performed using FloJo version 8.86 (Tree Star Inc.).

Depletion of regulatory T cells

Regulatory T cells (Tregs) were depleted using rat anti-mouse mAb directed against CD25 (clone: PC61) (gift from Dr. Christian Engwerda, QIMR) obtained from hybridoma supernatants as described previously [291]. Briefly, hybridoma cells were grown in RPMI-1680 2% NBCS with L-Glutamine, and penicillin/streptomycin (Invitrogen). Once fifty percent of the cells had died as visualised by light microscopy, supernatants were collected, pooled and tested for the presence of rat anti-mouse CD25 by flow cytometry. Each batch was titrated *in vivo* for their ability to deplete $CD4^+$, $FoxP3^+$ regulatory T cells. For depletion of Tregs during infection, typically 200-500μL containing ≈1mg of anti-CD25 or PBS was injected intra-peritoneally twice weekly for the duration of the experiment. PBMC were taken by venipuncture periodically to assess the degree of depletion as well as for T cell activation to ensure specificity of the depletion by flow cytometry. The course of disease was chartered weekly as a percentage increase in the size of the footpad lesion using a metric calliper (Kroeplin Schnelltaster, Schluechtern, Germany) and compared to the uninfected footpad. The percentage increase in footpad thickness was determined by the formula: Thickness of infected footpad/mean thickness of non-infected footpad x 100. The parasite burden was calculated as a proportion of tissue weight at day 28 after infection using a limiting dilution method and L-Calc software version 1.1 (Stem Cell Technologies) which performs a generalized Pearson Chi-squared test [112].

Cytokine Analysis.

Intracellular cytokine staining was performed on antigen-stimulated $CD4^+$ T cells. LN cells were re-stimulated using freeze-thawed *L. major* antigen (MOI equivalent = three) for 72 hours. For the last six hours of culture, the cells were cultured in the presence of 0.05μg/mL PMA, 1.35μM ionomycin (both purchased from Sigma-Aldritch) and 2.5μL monensin (Golgi-Stop, BD Biosciences) per sample in 2mL RPMI-1680 supplemented with 10% FCS, L-Glutamine, non essential amino acids, sodium pyruvate, penicillin/streptomycin and 2-mercaptoethanol (all supplied by Invitrogen). Subsequently, the cells were stained for surface antigens, fixed, permeabilised using FoxP3-FixPerm buffer (Biolegend) and stained for IFN-γ and IL-10 following the manufacturers instructions..

Peripheral blood was collected at different time points from infected mice and used to measure either haematocrit or blood glucose levels. For haematocrit, blood was drawn into heparinised capillary tubes to a length of 40-60mm (Pro-SciTech, Thuringowa) and sealed using Cristaseal plates as described by the manufacturer (Pro-SciTech) in triplicate for each animal. Capillary tubes were placed into FACS tubes and centrifuged at 2200 RPM at 4°C for 30 minutes. Haematocrit was calculated as the percentage of packed RBC's. Blood glucose was measured using Caresense Blood glucose test strips (i-SENS, Seoul, Korea) and measured using a Caresense II blood glucose meter (i-SENS).

Serum from peripheral blood was collected and cytokine concentrations were measured using mouse inflammation cytokine bead array (CBA, BD Biosciences), or human TGF-beta simple flex bead array. Acquisition of CBAs were performed using either a Cyan ADP (Beckman Coulter) or an ARIA II (BD Biosciecnes) digital cytometer and FCS 2.0

files were analysed using FCAP Array (Soft Flow Inc, Minnesota, USA). Alternatively IFN-γ concentrations were measured by an IFN-γ capture ELISA using anti-mouse IFN-γ capture (R4-6A2, BD Biosciences; 2μg/mL in carbonate buffer pH 9.6) and anti-IFN-γ biotin detection antibodies (XMG1.2, BD Biosciences) and developed using streptavadin horse radish peroxidase (BD Biosciences) and TMB liquid substrate (Sigma-Aldritch) and measured at 450nM following addition of 0.5M H_2SO_4. (Lomb Scientific, Brisbane).

Proliferation assays

$CD11c^+$, $CD8^-$ conventional dendritic cells (cDC) isolated were isolated from spleens of naïve mice. Briefly, spleens were minced and were digested with 1mg/mL collegenase D (Roche) in HBSS containing $Mg2^+$ and $Ca2^+$ (Invitrogen) at 37°C prior to passing through 60uM mesh to produce a single cell suspension. Cells were washed in PBS/0.1%BSA 2mM EDTA. Splenic cells were layered onto a 17.2%w/v Histodenz (Sigma Aldritch) in RPMI-1680 (Invitrogen) gradient and cells at the interface were subsequently purified by cell sorting with a FACS ARIA II (BD Biosciences), using a 100μm nozzle at 20 psi. Splenic DC were either $CD11c^+$, $CD11b^-$, $CD8^-$ or $CD11c^+$, $CD8^+$. Sorting purity was consistently >98%. $2x10^5$ cDC were pulsed with chicken Ovalbumin (Ova) peptide 323–339 (ISQAVHAAHAEINEAGR) overnight before addition of $5x10^5$ purified OT-2 Vβ5 TCR^+, $CD4^+$ T cells that had been labelled with 10μM CFSE (Invitrogen) as described previously [292]. Following 72 hours, cells were harvested and proliferation monitored by CFSE dilution by flow cytometry.

Gene Expression Analysis

Two-step Real Time PCR was performed on total RNA from purified $CD4^+$ naïve ($CD62L^+$ $CD44^-$) or $CD4^+$ activated ($CD62L^-$ $CD44^+$) splenic T cells from $1x10^6$ day 50 *L. major* infected mice. Cells were lysed using Trizol (Invitrogen) followed by RNA isolation using Purelink RNA micro kit (Invitrogen) as per the manufacturer's instructions. Synthesis of cDNA was performed using Superscript III following DNase treatment of RNA using RQ1 RNase free DNase (Promega). Gene expression was performed using Brilliant II SYBR green (Agilent Technologies, Integrated Sciences, Willoughby, NSW) and run on a Corbett Rotor Gene 6000 (Qiagen) and analysed using REST 2009 gene expression software (Qiagen) to determine relative expression of genes. PCR primers were designed using Vector NTI (Invitrogen): *β-actin, Hprt and Il-10* are described in Table 3.1 *Cblb* fw: GCA GCA TCA TTG ACC CTT TCA GCA, *Cblb*: rv: ATG TGA CTG GTG AGT TCT GCC TGT, *FasL* fw: GCA AAT AGC CAA CCC CAG TA, *FasL* rv: ATT CCA GAG GGA TGG ACC TT, *Ikaros* fw':CGG GAT CCC TTT GAG TGT AA, *Ikaros* rv: AGC TCA GGT GGT AAC GAT GC,

Statistics

Statistical analysis was performed using a non-parametric Mann Whitney test or a Kruskall-Wallace with Dunn's multiple comparison test to test for multiple hypotheses where appropriate. Examination of disease progression was analysed using a 2 way ANOVA with Bonferroni correction. Results are presented as mean values ± SEM or for gene expression as median ± S.E . Analyses were performed using GraphPad Prism 5.0 for

Macintosh (GraphPad Software). Each experimental group was compared to B6.WT controls. Statistical p values of $p \leq 0.05$ was considered to be significant with * $p \leq 0.05$ ** $p \leq 0.01$ and *** $p \leq 0.001$ respectively.

Results

TNF-deficient mice display increased T cell activation during leishmaniasis.

In chapter 3, I observed that mice deficient in TNF or TNFR1 fail to resolve infection with the protozoan parasite *L. major*. This occurs despite mounting a dominant Th1 type adaptive immune response accompanied by high levels of IFN-γ. To understand how the absence of TNF impacts on the generation of T cell responses in response to parasitic challenge I infected mice in the footpad with stationary phase *L. major* promastigotes and followed the course of $CD4^+$ T cell activation in B6.WT and B6.$TNF^{-/-}$ mice. TNF-deficient mice displayed consistently increased activation of $CD4^+$ T cells in both the draining pLN (Figure 4.1A) and the peripheral blood (Figure 4.1B) as shown by a decrease in CD62L expression and increased CD44 expression. This increase in activation was observed both relatively early in infection at day 21 where disease progression as measured by lesion development was similar to wild type, and at day 28 (Figure 4.1) where TNF-deficient mice were beginning to develop acute pathology (PDF and HK, (Figure 3.1). Despite a proportional increase in activation, quantification of absolute numbers showed no statistical differences in the numbers of activated and naïve $CD4^+$ T cells within the draining lymph node at day 21 (Figure 4.1A). At day 28 however, pLN from B6.$TNF^{-/-}$ mice showed significantly increased numbers of activated $CD4^+$ T cells and concurrent decrease in numbers of naïve $CD4^+$ T cells (See Figure 4.1B). Interestingly, despite the increased numbers of activated T cells, total T cell numbers within the pLN were reduced compared to wild type at day 28 which is also consistent with the observed reduced cellularity in the draining LN in the absence of TNF during later stages of infection (data not shown).

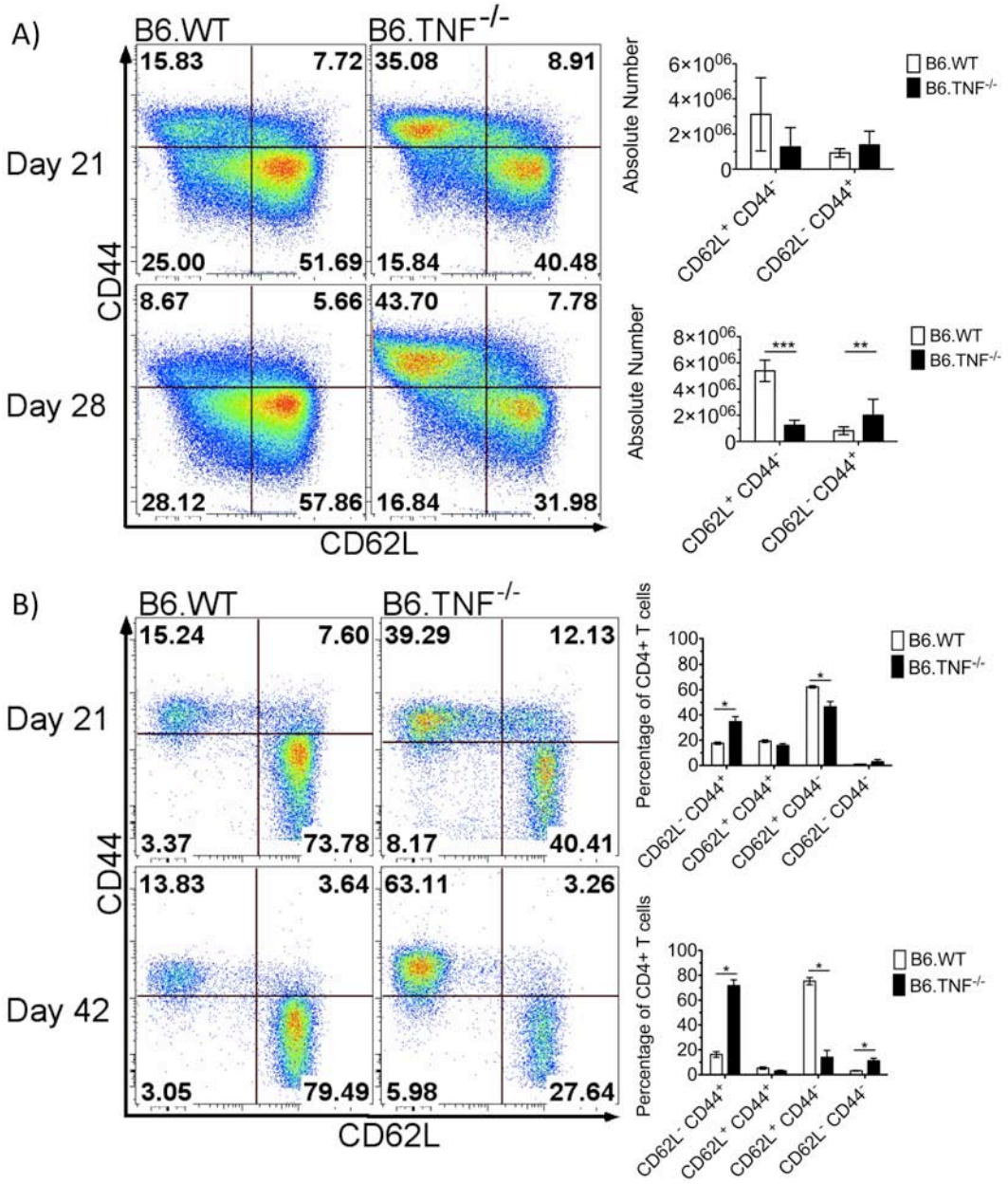


Figure 4.1 Infection with *L. major* results in increased CD4$^+$ T cell activation in the absence of TNF.

Flow cytometric analysis of CD4$^+$ T cell activation in the draining pLN (A) or peripheral blood (B) of B6.WT or B6.TNF$^{-/-}$ mice infected with 3x10^6 *L. major* promastigotes in the right hind footpad. CD4$^+$ T cells were analyzed for activation as measured by CD62L and CD44 expression and expressed as either absolute numbers in the pLN or as a percentage of CD4$^+$ T cells in peripheral blood. n= 4-5 mice per time point. * = $p<0.05$, *** = $p<0.001$. Statistical comparisons were performed using a Mann Whitney U test.

Previously I have shown that both wild type and TNF-deficient mice showed similar transcriptional and cytokine profiles consistent with an IFN-γ dominated $CD4^+$ T cell response (Figure 3.3). However, TNF signalling through TRAF and through NF-kappaβ is also involved in mediating the balance between survival and apoptosis [43, 293] as well as the maturation and differentiation of DC in both humans and mice [103, 294-296]. Therefore, I examined whether increased activation of $CD4^+$ T cells leads to an increase in anergy or in activation induced cell death. Expression of *FasL* was significantly up-regulated in $CD4^+$, $CD44^+$, $CD62L^-$ T cells in TNF-deficient mice by a median factor of 6.7-fold compared to wild type mice that had median induction of 5-fold compared to naïve $CD4^+$ T cells. Interestingly, the expression of the transcriptional regulator *Ikaros* (*Ikzf1*), which has been shown to be involved in chromatin remodelling of the IL-2 locus during anergy induction and in repressing the transcription of *Tbx-21* and *Ifng* [297] was up-regulated in activated $CD4^+$ T cells from B6.$TNF^{-/-}$ $CD4^+$ T cells by 2.7-fold and was unchanged in activated B6.WT $CD4^+$ T cells. The E3 ubiquitin ligase *Cbl-b*, which is also involved in the induction of anergy [298] remained unchanged compared to naïve $CD4^+$ T cells in both B6.WT and B6.$TNF^{-/-}$ $CD4^+$ T cells (See Figure 4.2A).

To ascertain whether any increase in AICD was due to T cell: T cell interaction or due to dendritic cell: T cell interactions such as those described for the control of $CD8^+$ T cells in viral infections [299] I examined the contribution of TNF and FasL on splenic cDC to modulate proliferation and survival of $CD4^+$ T cells. Transgenic Vβ5 TCR^+ OT-II $CD4^+$ T cells were co-cultured with either $CD11c^+$, $CD8^-$ or $CD11c^+$, $CD8^+$ splenic cDC from either B6.WT, B6.$TNF^{-/-}$ or B6.TNF/$FasL^{-/-}$ mice that had been pulsed previously

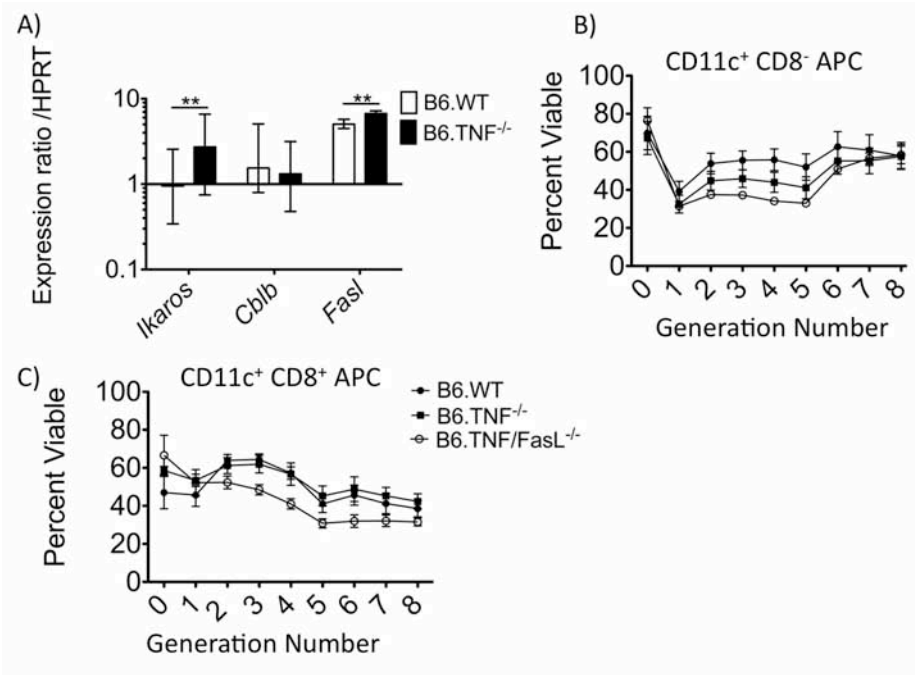


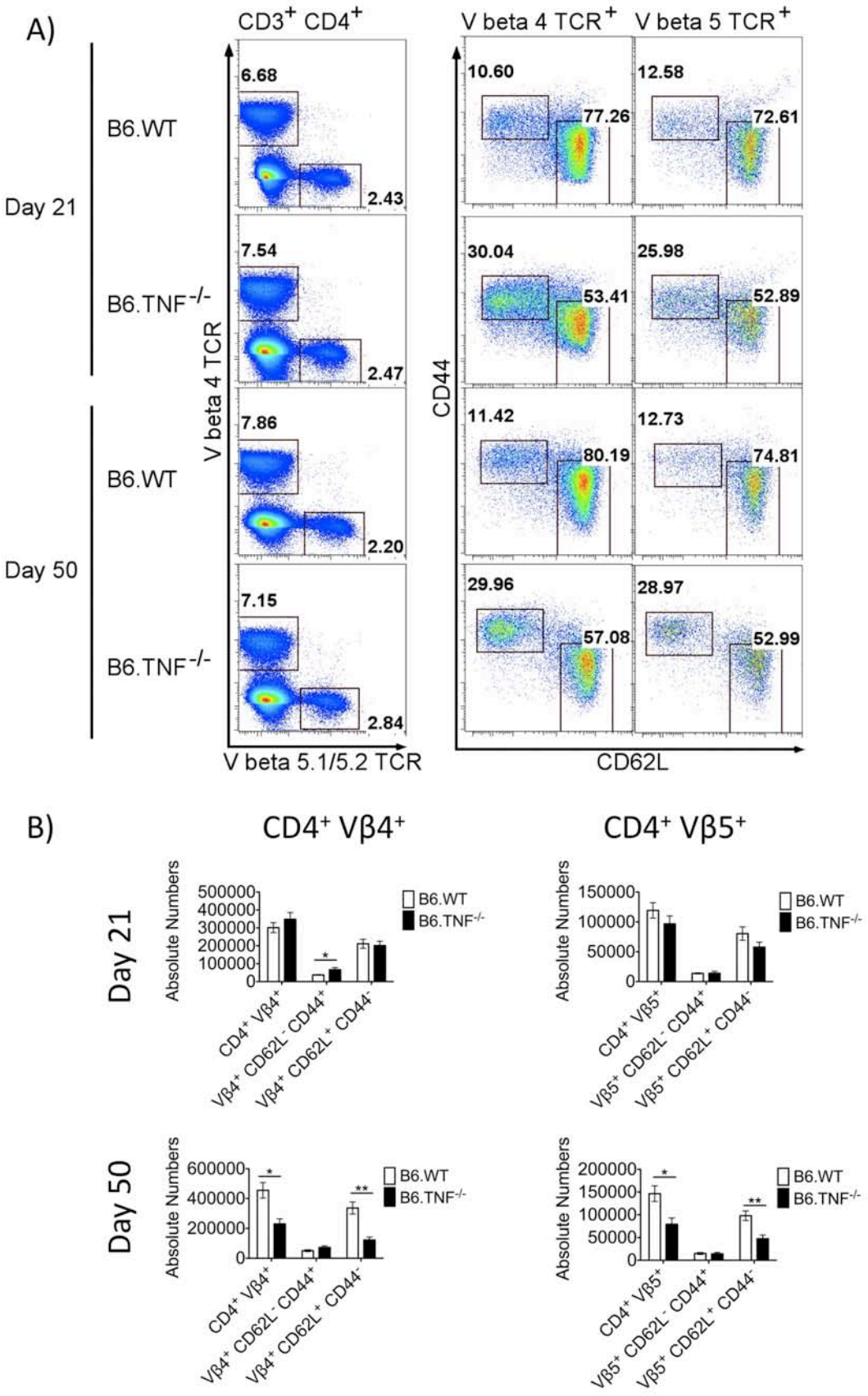
Figure 4.2 Increased *FasL* and *Ikaros* expression in TNF-deficient activated CD4$^+$ T cells indicative of activation induced cell death.

Real time PCR was performed to detect genes associated with activation induced cell death (A) from purified day 50 *L. major* infected activated CD4$^+$, CD62L$^-$, CD44$^+$ T cells and compared to naïve CD4$^+$, CD62L$^+$, CD44$^-$ from the same animal. Differences in gene expression between B6.WT and B6.TNF$^{-/-}$ naïve (CD62L$^+$, CD44$^-$) is shown. Gene expression is shown as the median +/- S.E relative to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), n =5-6. Statistical analysis was performed using REST 2009 analysis software (Qiagen) and statisitical comparisons were performed using bootstrap randomization * p≤ 0.05 ** p≤0.01. (B) Induction of activation induced cell death. 5x10^5 CFSE labeled Vβ5$^+$ OT II CD4$^+$ T cells were co$^-$cultured with either 5x10^4 purified CD11c$^+$, CD8$^-$ (B) or CD11c$^+$, CD8$^+$ (C) splenic DC from either B6.WT (n=7), B6.TNF$^{-/-}$ (n=5) or B6.TNF/FasL$^{-/-}$ (n=3) mice that had been pulsed with 1μg/mL Ova-peptide overnight. Cells were harvested after 90 hrs and proliferation measured by CFSE dilution using a FACS Aria II. Propidium iodide exclusion was used to detect viable cells at each proliferation peak. Data is displayed as the mean +/- S.E.M.

overnight with chicken OVA peptide 323–339 (ISQAVHAAHAEINEAGR). These cDC populations both induced robust proliferation in $CD4^+$ OT-II T cells. Interestingly, analysis of T cell survival by propidium iodide exclusion at each cell division showed no statistical difference in division linked viability induced by either B6.WT or B6.TNF splenic DC although TNF/$FasL^{-/-}$ cDC showed a decrease in viability compared to either B6.WT or B6.$TNF^{-/-}$ (See Figure 4.2 B and C).

Early expansion of Vβ4 and Vβ5 TCR restricted $CD4^+$ T cells in the absence of TNF during cutaneous leishmaniasis.

The high degree of activation observed in $CD4^+$ T cells from TNF-deficient mice during infection with *L. major* prompted an examination of the dynamics of T cell activation with regards specific TCR usage. Both susceptible and resistant strains of mice develop strong antigen specific responses to the LACK antigen with a dominant Vα8 Vβ4 restricted TCR repertoire [300]. I followed the expansion and activation of Vβ4 TCR^+, $CD4^+$ T cells in both B6.WT and B6.$TNF^{-/-}$ mice and concurrently compared it to Vβ5.1/5.2$^+$ TCR^+, $CD4^+$ T cells that do not expand normally in response to infection with *L. major* [283]. At day 21 post infection, both B6.WT and B6.$TNF^{-/-}$ mice showed similar proportions and absolute numbers of total Vβ4, $CD4^+$ T cells within the draining LN. However, Vβ4 TCR^+, $CD4^+$ T cell from B6.$TNF^{-/-}$ mice showed a significant increase in activation as measured by increased CD44 expression, which is also reflected by an increase in the absolute numbers of activated Vβ4$^+$, $CD4^+$ T cells within the draining pLN (Figure 4.3 A and B).


A)
CD3+ CD4+
V beta 4 TCR+
V beta 5 TCR+
Day 21
B6.WT
B6.TNF-/-
Day 50
B6.WT
B6.TNF-/-
6.68
2.43
7.54
2.47
7.86
2.20
7.15
2.84
10.60
77.26
12.58
72.61
30.04
53.41
25.98
52.89
11.42
80.19
12.73
74.81
29.96
57.08
28.97
52.99
V beta 4 TCR
V beta 5.1/5.2 TCR
CD44
CD62L
B)
CD4+ Vβ4+
CD4+ Vβ5+
Day 21
Day 50
Absolute Numbers
B6.WT
B6.TNF-/-
CD4+ Vβ4+
Vβ4+ CD62L- CD44+
Vβ4+ CD62L+ CD44-
CD4+ Vβ5+
Vβ5+ CD62L- CD44+
Vβ5+ CD62L+ CD44-

Comparison of Vβ5$^+$, CD4$^+$ T cells at day 21 showed both similar proportions and absolute counts between B6.WT and B6.TNF$^{-/-}$ mice with greatly reduced numbers compared to

Vβ4$^+$ T cells, indicative of failure to respond to *Leishmania* antigen. Interestingly Vβ5$^+$, CD4$^+$ T cells from B6.TNF$^{-/-}$ mice showed a similar increase in activation to Vβ4$^+$, CD4$^+$ T cells, which was not observed in B6.WT mice. Despite the similar numbers of total Vβ4$^+$ and Vβ5$^+$, CD4$^+$ T cells observed at day 21, by day 50 post infection B6.TNF$^{-/-}$ mice had significantly reduced numbers of both total pLN (data not shown) and Vβ4$^+$ and Vβ5$^+$, CD4$^+$ T cells subsets. This reduction in absolute numbers correlated with the observed proportional increase in activation similar to that observed at day 21. Quantification, however, revealed no significant difference in the numbers of activated Vβ4$^+$ and Vβ5$^+$ CD4$^+$ T cells at this later time point, but instead, show that the proportional difference in activation results from a decrease in naïve CD62L$^+$ CD44$^-$ T cells from the pLN irrespective of their TCR V beta usage during infection (See Figure 4.3B).

This lead me to examine whether the observed increase in CD4$^+$ T cell activation and the associated expansion of typically non-dominant Vβ TCR chains early in infection resulted specifically from differential TNFR signalling. Consistent with previous experiments, mice deficient in TNF revealed an expansion of Vβ4$^+$ and Vβ5$^+$ CD4$^+$ T cells that were

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Figure 4.3 Increased T cell activation is associated with an increase in epitope spreading.

Mice were infected with 3x10^6 *L. major* promastigotes in the hind footpad and lymph nodes were collected and analysed at day 21 and 50 post infection. (A) CD3$^+$ CD4$^+$ T cells were analyzed for expression of Vβ4 and Vβ5 TCR chains in conjunction with CD62L and CD44 to monitor activation (B) Quantitation of absolute numbers of naïve (CD62L$^+$ CD44$^-$) and activated (CD62L$^-$ CD44$^+$) CD4$^+$ Vβ4$^+$ and Vβ5$^+$ CD4$^+$ T cells in the draining pLN at day 21 and day 50 post infection. n= 8 (B6.WT) or 9 (B6.TNF$^{-/-}$) and is representative of 3 independent experiments. * = p≤0.05 ** p≤0.01 Statistical comparisons were performed using a Mann Whitney U test.

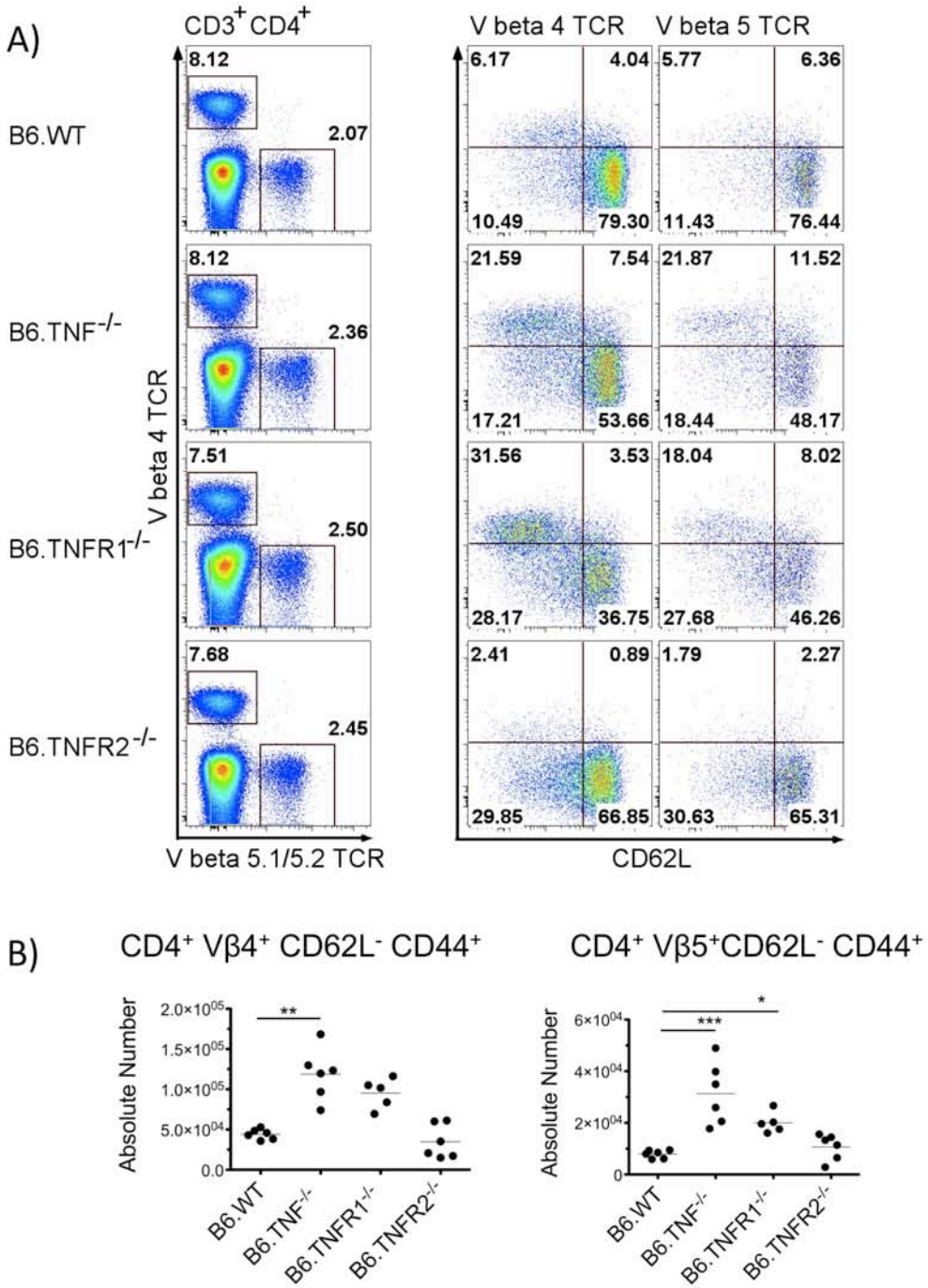

Figure 4.4 Expansion and activation of Vβ TCR repertoire occurs in the absence of TNF or TNFR1 but not TNFR2.

B6.WT or mice lacking TNF, TNFR1 or TNFR2 were infected with $3x10^6$ *L. major* promastigotes and draining pLN harvested at day 21 post infection. B6.TNF and B6.TNFR1-deficient mice showed increased activation of both $CD4^+$ T cells expressing either Vβ4 or Vβ5 TCR chains (A). Increased activation was correlated with increased absolute numbers of both Vβ4 or 5 restricted activated ($CD62L^-$ $CD44^+$) $CD4^+$ T cells in the draining pLN (B). Statistical comparisons were performed using a Kruskal Wallis Test with Dunns correction n= 5-6 * = p≤0.05 ** p≤0.01, *** = p<0.001. One of two representative experiments is shown.

phenotypically activated at day 21 post infection as measured by increased CD44 expression (Figure 4.4A and B). This increased expansion and activation was paralleled in mice lacking TNFR1 but not TNFR2, with associated increases being observed in both $V\beta4^+$ $CD4^+$ T cells representative of a polyclonal cell population containing LACK-reactive T cells, as well as non typical $V\beta5^+$ $CD4^+$ T cells (Figure 4.4 A and B).

Expansion of Regulatory T cells during chronic infection

The discontinuity in the observed increase in IFN-γ production and increased T cell activation that accompanies the establishment of chronic infection in the absence of TNF and TNFR1, which ultimately leads to death following infection with *L. major,* prompted me to look at the possible immuno-suppressive mechanisms that may contribute to uncoupling of innate and acquired immune responses. Following infection with *L. major* promastigotes, both B6.$TNF^{-/-}$ and B6.$TNFR1^{-/-}$ mice began to show increased numbers of $CD4^+$, $FoxP3^+$, $GITR^+$ Tregs that peaked at between day 21 and day 28 and remained elevated for the remainder of the observed period of infection (Figure 4.5 A and B). It is interesting that the observed peak of Treg accumulation corresponded with the divergence in disease progression from B6.WT mice. This expansion of Tregs in the absence of TNF was in contrast to B6.WT mice that showed an early increase that peaked at day 21 and began to subside and correlated with resolution of the infection. B6.TNFR2-deficient mice showed only minimal changes in the accumulation of Tregs over the entire course of disease. However, despite the observed trend towards increased numbers of Tregs in both TNF and TNFR1-deficient mice, comparison of the ratio of regulatory T cells to absolute $CD4^+$ T cells showed no positive correlation between any of the genotypes indicating that

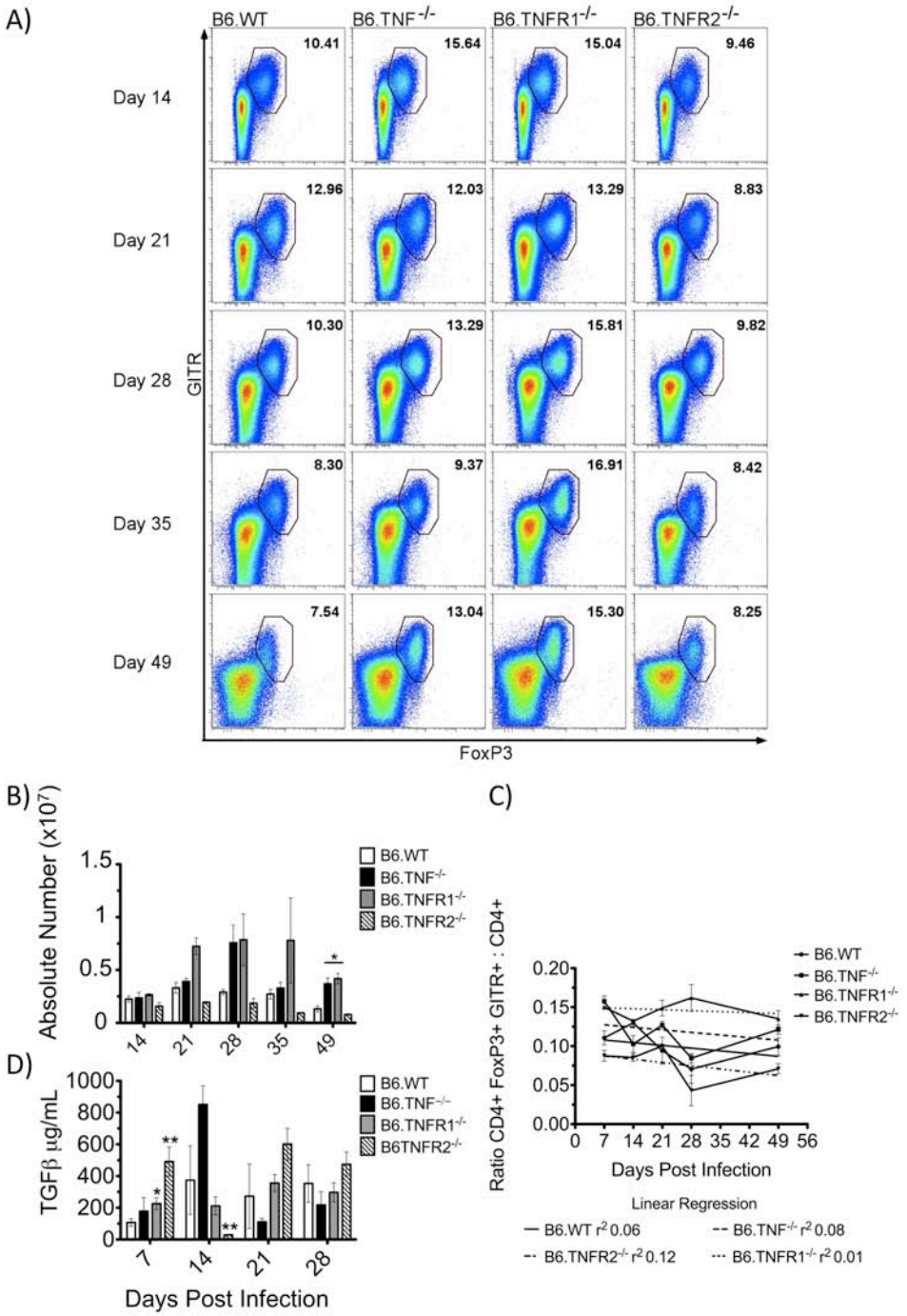

Figure 4.5 Expansion of Regulatory T cells in the absence of TNF or TNFR1 during experimental cutaneous Leishmaniasis.

B6.WT, B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$ and B6.TNFR2$^{-/-}$ mice were infected with *L. major* promastigotes and the numbers of Splenic CD4^{+} FoxP3^{+} GITR^{+} Tregs cells monitored throughout the course of disease. B6.TNF$^{-/-}$ and B6.TNFR1$^{-/-}$ mice show proportional increases in the numbers of Tregs (A) and in absolute numbers (B), which reached significance accounting for multiple hypothesis testing at day 49 post infection. n= 3-4 mice. Data presented is one of two independent experiments (C) TGFβ was measured at weekly intervals by cytometric bead array from serum of *L. major* infected B6.WT, B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$ and B6.TNFR2$^{-/-}$ mice. n=5 Statistical comparisons were performed using a Mann Whitney U test * = p≤ 0.05.

the expansion of Tregs observed in B6.TNF$^{-/-}$ and B6.TNFR1$^{-/-}$ mice may result from a linear development that parallels normal CD4^{+} T cell expansion during the chronic infection (Figure 4.5 C).

To asertain if this increase in Tregs resulted promoted a supressive cytokine mileau I analysed serum TGF-β levels during the first 4 weeks of *L. major* infection. TGF-β levels

showed only small differences between all genotypes early in the infection. Both B6.TNFR1 and B6.TNFR2-deficent mice showed peak enhanced serum TGF-β concentrations at day 7 post infection compared to B6.WT, while B6.WT and B6.TNF$^{-/-}$ both peaked at day 14 post infection. All genotypes showed similar TGF-β levels at day 21 and day 28 post-infection (See Figure 4.5 D).

To see if the observed increase in Tregs in B6.TNF-deficeint mice impacted on the cytokine profile of the CD4^{+} T cell response I looked at both IFN-γ and IL-10 production by CD4^{+} T cells. Both B6.WT and B6.TNF$^{-/-}$ CD4^{+} T cells produced IL-10 early in the infection, but it quickly became dominated by IFN-γ (See Figure 4.6A). Similar to my previously reported findings (Figure3.2), B6.TNF$^{-/-}$ mice showed enhanced CD4^{+} T cell IFN-γ production from day 14 onwards (Figure 3.2). Interestingly, while early IL-10 production was associated with both IFN-γ-producing and non-producing CD4^{+} T cells, during later time points B6.TNF$^{-/-}$ mice showed a small but significantly sustained expression of IL-10 that was co-expressed with IFN-γ (Figure 4.6A and B). While there appeared to be an increased ability for B6.TNF$^{-/-}$ CD4^{+} T cells to produce IL-10, this did not appear to be produced by natural CD4^{+} FoxP3^{+} CD25^{+} regulatory T cells but rather from a CD4^{+} CD44^{+} CD25^{-} population and mirrored the IL-10 producing cells observed in B6.WT mice (data not shown).

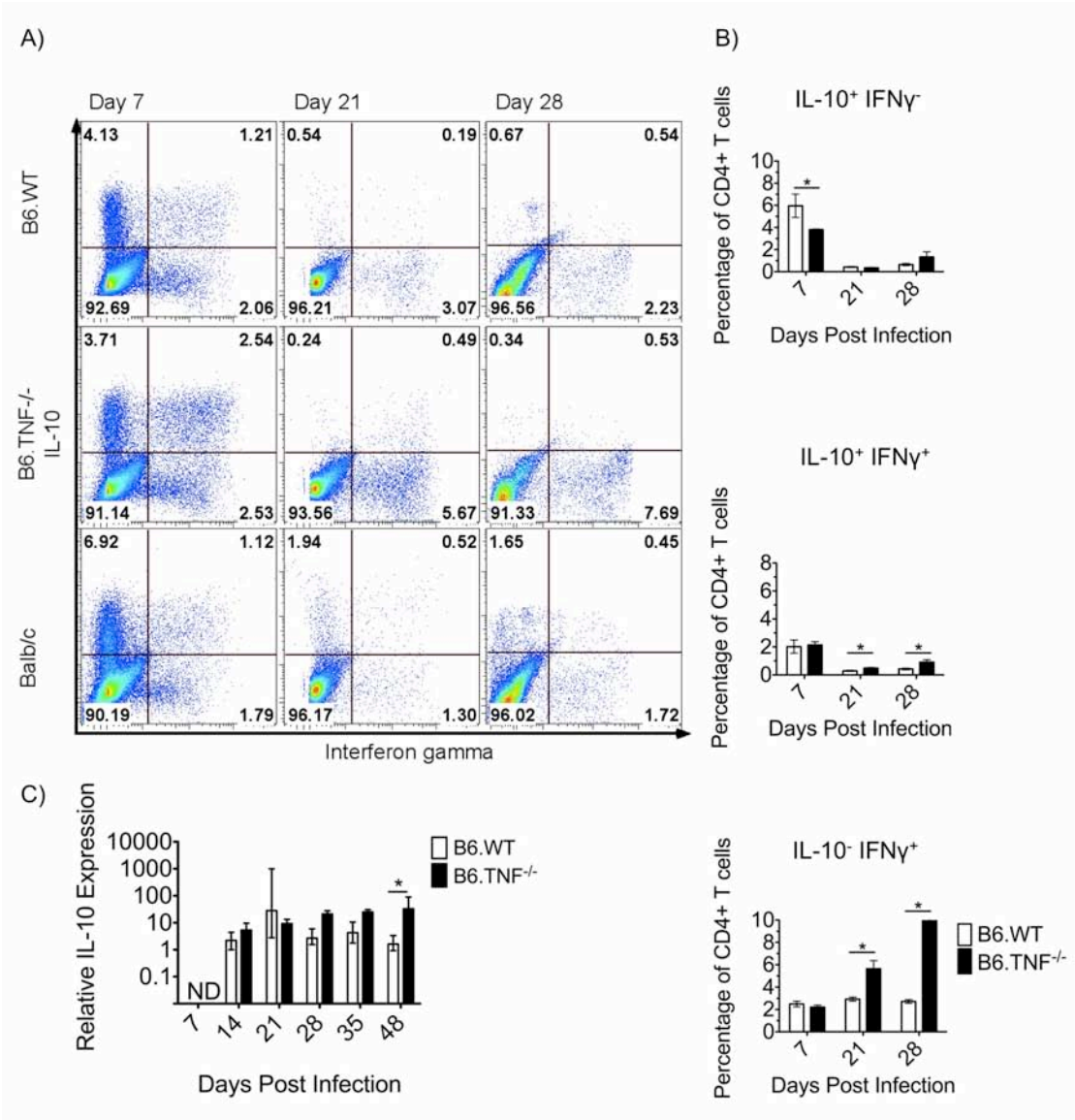

Figure 4.6 Early increase in IL^{-}10 production by CD4^{+} T cells during infection with *L. major*.

A) Intracellular flow cytometry was used to determine the expression of *IFN-γ* and *Il-10*. A representative bivariate plot is shown. B) The proportions of IFN-γ^{-} IL-10^{+} (UL), IFN-γ^{+} and IL-10^{+} (UR), IFN-γ^{+} and IL-10^{-} (LR) are given as a mean (± SEM, n = 4, * = $p<0.05$). C) IL-10 expression was concurrently measured in the footpad lesion of *L. major* infected mice over the course of the disease. Relative expression compared to *B-actin* is shown as the median ± S.E and was calculated using REST 2009 software (Qiagen) n=5 The experiment was performed two times independently.

To test if the accumulation of natural Tregs observed in the absence of TNF or TNFR1 impacted on the course of cutaneous leishmaniasis, I endeavoured to deplete Tregs through the administration of anti-CD25 (clone, PC61) mAb. Since CD25 is also present on activated T cells I assessed both the presence of $CD25^+$ T cells using anti CD25 (clone, 7D4) mAb and the levels of T cell activation in both B6.WT and B6.$TNF^{-/-}$ mice throughout the course of infection in treated and control mice. Depletion of Tregs in either

B6.WT or B6.mem$TNF^{\Delta/\Delta}$ mice did not alter disease progression ($p>0.05$) from the untreated controls (Figure 4.7A) similar to results published previously [113]. Interestingly, TNF, TNFR1 and TNFR2- *L. major*-infected deficient mice treated with anti-CD25 mAb (PC61) showed similar courses of disease compared to untreated and resulted in similar pathology, where both TNF and TNFR1-deficient mice succumbed to disease while TNFR2-deficient mice survived the infection but failed to heal the lesion. Notably, the failure to control the infection was not due to inadvertent depletion of activated T cells. Both B6.WT and B6.$TNF^{-/-}$ mice showed substantial loss of $CD4^+$ $CD25^+$ T cells from peripheral blood at both day 21 and day 42 post infection, while maintaining similar $CD4^+$ T cell activation profiles to the untreated controls during the course of *L. major* infection (Figure 4.7B)

Depletion of regulatory T cells by anti-CD25 mAb did not result in any notable reduction in parasite burden in either the footpad lesion of in the spleen as a consequence of visceralisation in the absence of TNF (Figure 4.8A). Neither did it affect the production of the serum cytokines IFN-γ or MCP-1 or affect total haematocrit or blood glucose levels (Figure 4.8 B-E), negating any influence of Treg depletion in mediating immunopathology associated with infection.

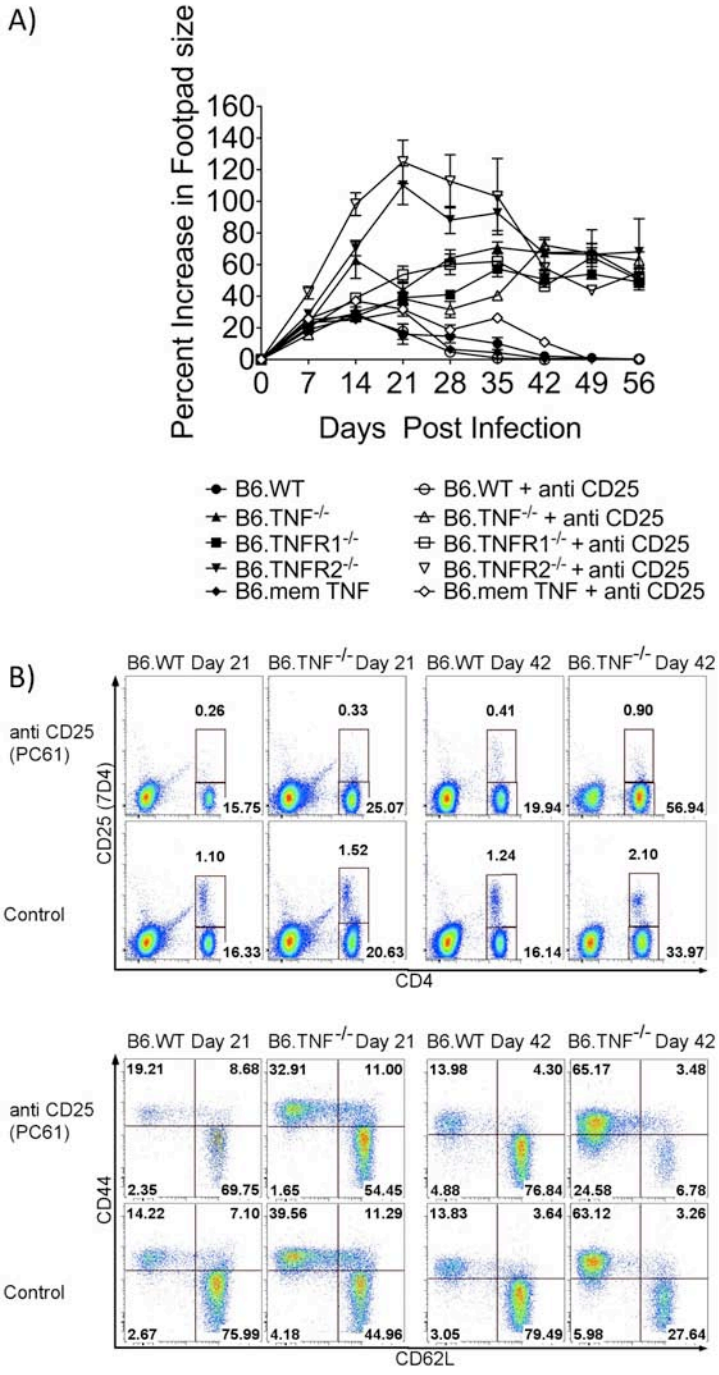

Figure 4.7 Depletion of regulatory T cells does not ameliorate disease progression during infection with *L. major* in the absence of TNF.

A) Course of disease in B6.WT, B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$, B6.TNFR2$^{-/-}$, and memTNF$^{\Delta/\Delta}$ with (open symbols) and without (closed symbols) depletion of regulatory T cells. Tregs were depleted by i.p injection of anti-CD25 mAb (PC61) twice weekly throughout the course of infection with *L. major*. Differences were evaluated using a 2-way Anova with Bonferonni correction. B) Effectiveness of Treg depletion was monitored at day 21 and 42 by flow cytometry using anti CD25 (7D4), additionally activation of CD4^{+} T cells (CD44^{+} CD62L^{-}) was monitored at the same points to ensure the specificity of depletion. n=4-6 per genotype per treatment. One of three independent experiments is shown.

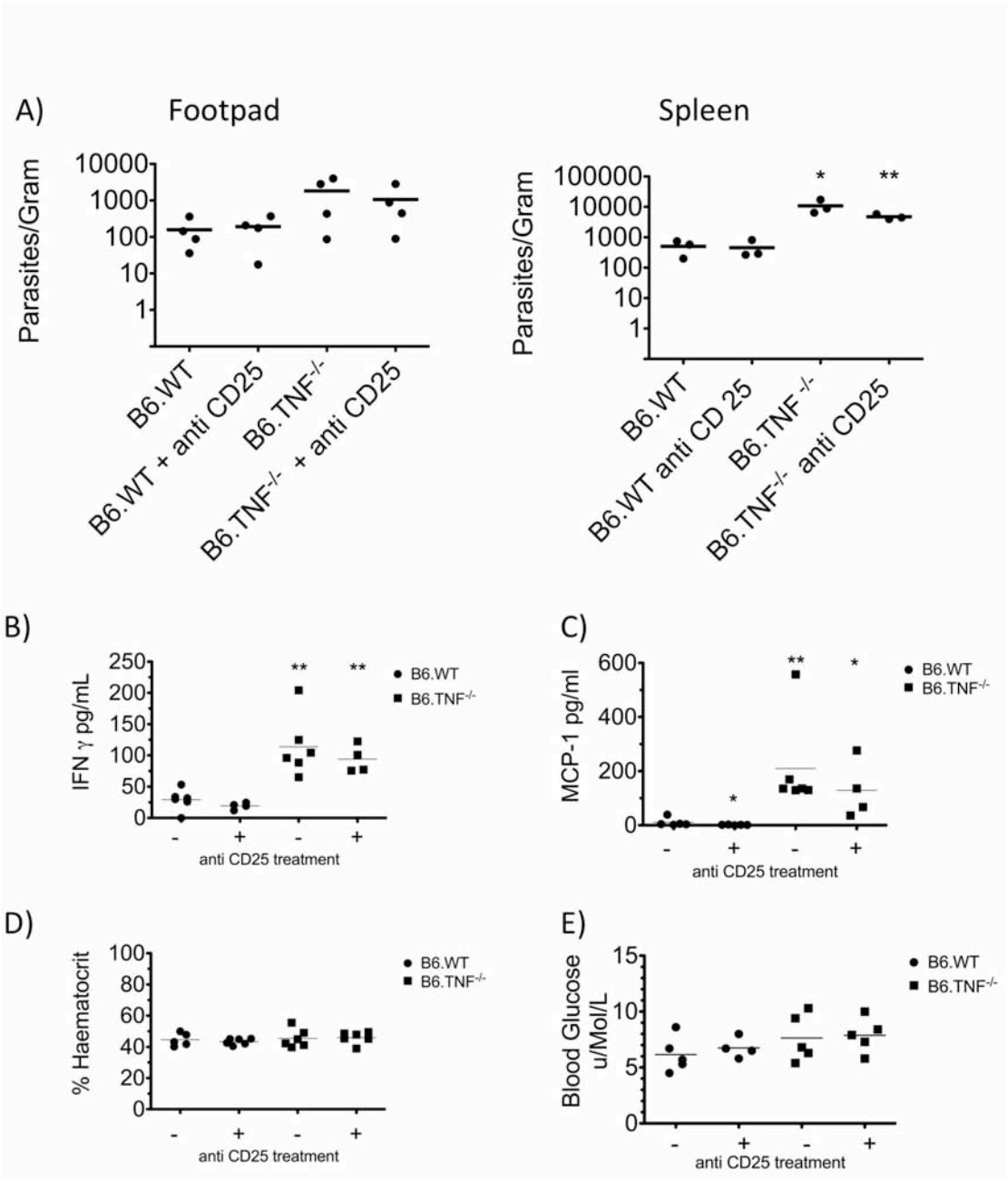

Figure 4.8 Depletion of regulatory T cells in the absence of TNF fails to control parasite dissemination.

A) Parasite burdens were determined from footpad lesions and spleens at day 28 post infection of control B6.WT or B6.TNF$^{-/-}$ mice or mice that had received anti-CD25 (PC61) mAb i.p twice weekly. n=3-4 mice. Concentrations of IFN-γ (B) and MCP-1 (C) were determined from serum of anti-CD25 treated or control mice at day 28 post infection n= 4-6 mice. Haematocrit (D) and blood glucose levels (E) were measured at day 56 post infection n= 4-6 mice. One of two independent experiments is shown. * =p≤ 0.05, ** =p≤ 0.01.

Discussion

The delineation of Th1 and Th2 responses in resistance and susceptibility to experimental cutaneous leishmaniasis has provided a substantial contribution in understanding the molecular basis of T cell differentiation in the context of infectious disease. I have shown previously, that dissociation of protective IFN-γ responses occur during infection with *L. major* in the absence of TNF, despite the generation of a classical IFN-γ Th1 type response and results in disease susceptibility (Chaper 3) [112]. Resolution of cutaneous leishmaniasis in resistant mice has been shown to encompass both the effector and regulatory arms of the adaptive immune response. IFN-γ producing $CD4^+$ effector T cells are important in driving parasite clearance through the activation of leishmanicidal functions cells of the innate system [244, 301, 302]. Conversely, regulatory T cells suppress inflammation and in the context of diseases such as leishmaniasis and promotes a state of concomitant immunity that facilitates the persistence of parasites at sites of infection and appears critical to the establishment of memory responses following re-infection [291, 303]. This suppressive ability, in the context of *L. major* infection, is thought to be strongly dependent on IL-10 produced by natural $CD4^+$, $CD25^{+,}$ $FoxP3^+$ Tregs that facilitate the maintenance of parasites following clinical cure. This is especially highlighted in a number of experiments utilizing IL-10R-deficient mice or following administration of anti-IL10 mAb or the adoptive co-transfer of $CD4^+$, $CD25^-$ and $CD4^+$, $CD25^+$, $IL10^{-/-}$ T cells into RAG-deficient mice, all of which result in the establishment of sterile cure [101, 291, 303]. However, another study has questioned the role for natural Treg derived IL-10 in parasite persistence and show a role for $CD4^+$, $FoxP3^-$, $CD25^-$ IL-10 production in chronic non-curing *L. major* (MHOM/SN/74/SD) in IFN-γ responding mice

[304]. A perturbment in equilibrium between effector and regulatory T cells has also been shown by Mendez and colleagues to be involved with reactivation of latent infection following re-infection at distal sites, resulting from accumulation and recruitment of Tregs to sites containing latent parasites [291].

Unlike B6.WT mice, T cell activation is greatly increased in the absence of TNF, however enhancement of activation as measured by increased CD44 expression does not reflect positively on the clinical outcome. The expansion of highly activated IFNγ^+ CD4$^+$ T cells during the course of infection is accompanied by a total reduction in the cellularity of the lymph nodes while concurrently resulting in systemic inflammatory sequelae such as hepato-splenomegaly. The accompaniment of the reduction in the total cellularity of the lymph nodes, by lymph node atrophy reflects at least in the T cell compartment a loss of naïve (CD62L$^+$, CD44$^-$) T cells while maintaining the presence of activated T cells (CD62L$^-$, CD44$^+$). This may reflect a state of chronic local inflammation that ultimately results in immunopathology resulting in pro-apoptotic conditions that favours the survival of activated T cells.

The increase in the activation of Vβ5$^+$ TCR chain CD4$^+$ T cells in the absence of TNF, which reflects activation to putative minor *Leishmania* spp. epitopes not normally expanded in B6.WT [283], also suggests a state of chronic inflammation, resulting in bystander activation or epitope spreading during the acute phase of infection. The exact contribution of this epitope spreading in the context of leishmaniasis and immunopathology is still to be determined. It is interesting to note that this observed expansion of Vβ5$^+$ TCR T cells is most notable early in infection and that this expansion is masked during late chronic stage infection possibly due to lymph node pathology.

Similarly, it remains to be seen if these surviving activated T cells found in the lymph node directly contribute to pathology through their reactivity to non-canonical epitiopes or whether they may contribute directly to dissociation of IFN-γ responses from leishmanicidal activity.

However, despite the increased $CD4^+$ T cell activation observed in the absence of TNF and TNFR1, a burst of IL-10 is produced by $CD4^+$ T cells in both B6.WT and B6.$TNF^{-/-}$ mice and this is coincident with IFN-γ expression in the draining lymph node. While, this appears to be only slightly elevated in TNF-deficent mice, a sustained increase in IL-10 expression is observed in the footpad lesion of B6.$TNF^{-/-}$ mice during later time points, which may belie an imbalance in the regulation of the effector phase of the immune response. The induction of IL-10, despite the ongoing IFN-γ response, may still provide a means to limit immune activation especially in MΦ which serve as a major reservoir of *L. major* amastigotes *in vivo*. In fact, IL-10 has been shown to be able to promote parasite persistence in normally resistant strains of mice through modulating the recruitment of cells to the site of inoculation and promoting a permissive environment in the draining lymph node [305]. The ability of IL-10 to modulate MΦ function and suppress IFN-γ activating effects including antigen presentation and direct anti-microbial effector functions such as ROS and possibly RNI [242] may be sufficient to unlink IFN-γ production from leishmanicidal functions.

While I have observed a similar expansion of regulatory T cells during the chronic phase of cutaneous leishmaniasis, this increase is markedly enhanced in the absence of TNF or TNFR1. It is interesting, in light of the equilibrium hypothesis proposed by Mendez et al., in which both effector and regulatory T cells expand in response to rechallenge but differ

in response to migratory patterns allowing for both immunity and reactivation [291], that the large expansion of Tregs I observed appears to coincide with the diversification of the immune response from cutaneous to a visceral form. This transition from cutaneous to visceral leishmaniasis observed in B6.TNF$^{-/-}$ and B6.TNFR1$^{-/-}$ mice may correspond to the distal rechallenge described by Mendez et al., and allow the expansion of both effector and regulatory CD4^{+} T cells [291]. This is consistent with my data, which shows a linear expansion of Tregs that follows an increase in total CD4^{+} T cells. It is interesting, however, that the observed systemic expansion of CD4^{+}, FoxP3$^{+,}$ GITR^{+} Tregs in the absence of TNF or TNFR1 does not appear to be directly linked to the fatal outcome observed in these mice, as CD25 depletion using PC61 mAb did not result in systemic differences in parasites burdens or cytokine responses. It is plausible that the parasitemia and chronic inflammation observed may simply overwhelm the host and mask the contribution of Tregs in this instance.

Chapter 5: Changes in the inflammatory monocytic response in rapidly fatal experimental cutaneous leishmaniasis in the absence of TNF.

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Introduction

The model of experimental cutaneous leishmaniais has provided a useful tool to study the genetics that control susceptibility to infection with *L. major*. A large body of evidence exists that the protective immune response is characterised by the production of IFN-γ by $CD4^+$ T cells [68, 271] and conferred by the induction and action of the major innate effector molecule nitric oxide (NO) [244, 257]. A role for TNF in resistance to intracellular pathogens such as *L. major* has been demonstrated in experimental models [270] but the mechanisms by which this cytokine exerts its protective effects remain undefined. The absence of TNF or TNFR1 confers subtle alterations in the microarchitecture of the secondary lymphoid organs [5] primarily through the modulation of expression of basal homing chemokines such as CCL21 and CXCL13 [6] which regulate the migration of cells such as T cells and activated dendritic cells into their respective compartments of spleen and LN. Experiments utilising reciprocal bone marrow chimeras have helped to exclude a major role of for structural deficiencies in the susceptibility of B6.$TNF^{-/-}$ mice and have established the exclusive activity of hematopoietically derived TNF in contributing to a protective immune response [112].

TNF has been identified *in vitro* as a strong co-stimulatory signal that facilitates the sustained induction of the iNOS promoter in murine bone marrow macrophages [106, 227, 229, 306-309]. While TNF is produced by different cells types including $CD4^+$ and $CD8^+$ T cells [63], MΦ [21] and NK cells [23] a group of inflammatory myeloid cells has been identified recently as major source of TNF. These cells have been termed TNF/iNOS producing dendritic cells (TIP-DC) [310], monocyte derived dendritic cells (Mo-DC) [311], inflammatory dendritic cells [312] or inflammatory monocytes [313, 314] and are

characterized by their expression of the CC-chemokine receptor CCR2 and Ly6C. The importance of these cells in the context of infection is highlighted by the consequences of a loss of inflammatory monocytes from inflamed tissues in mice deficient in the chemokine receptor CCR2, which show exacerbated disease progression and increased mortality in a number of infection models including *L. monocytogenes* [315, 316] *T. gondii* [317, 318] and *L. major* [319].

Therefore, I hypothesize that the actions of TNF in regulating cellular migration as well as inducing differentiation and, ultimately, effector functions in innate myeloid cells recruited to sites of infection are likely to contribute to the susceptibility seen in mice lacking either TNF or TNFR1. I therefore examined the role of TNF on inflammatory monocytes during experimental cutaneous leishmaniasis.

Materials and Methods

Mice

The gene-targeted C57BL/6 mouse strain deficient for soluble and membrane TNF (B6.TNF$^{-/-}$) or for soluble TNF (B6.memTNF$^{\Delta/\Delta}$) only was generated on a genetically pure C57BL/6 (B6.WT) background as described [5, 19]. The B6.TNFR1$^{-/-}$ (Jackson stock number: 003242) and B6.TNFR2$^{-/-}$ mice (Jackson stock number: 002620) were obtained from Jackson Laboratories and had been backcrossed more than 10 times or had been established on a C57BL/6 background, respectively [265]. The screening procedure followed the protocols published previously [5, 265]. All animals were kept under specific pathogen free conditions at the Animal Research Facilities of the Comparative Genomics Centre. All experiments followed protocols approved by the animal ethics committee of James Cook University, Townsville, Australia. Mice of 8-12 weeks of age were used in all experiments.

Parasites and infection

The virulent *L. major* isolate MHOM/IL/81/FE/BNI or the eGFP expressing, transfected *L. major* isolate MHOM/IL/80/Friedlin-eGFP [290] (a gift from Dr. Emanuela Handman, Walter and Eliza Hall Institute, Melbourne, Australia) was maintained through serial passage in BALB/c mice *in vivo* and cultured *in vitro* in Novy-Nicolle-MacNeal blood agar slants in RPMI containing 10% new born calf serum, Penicillin/Streptomycin, Non Essential Amino acids and 10mM HEPES [266], all supplied by Invitrogen. Additionally,

eGFP transgenic *L. major* parasites were cultured in the presence of 10μg/mL Hygromycin B (Invitrogen) to postitively select for eGFP expression. For infection, stationary phase *L. major* promastigotes were used between passage two and six and $3x10^6$ parasites were injected in a volume of 40μl into one hind footpad. For *in vitro* assays purified Mo-DC's were cultured in 8 or 16 well chamber slides (Pro-Sci-Tech) and infected with *L. major* promastigotes (MOI = three) or stimulated with Lipopolysaccharide (LPS)(Sigma-Aldritch). Alternatively monocyte populations were isolated *ex situ* from *L. major* infected mice and cultured overnight in chamber slides. Microscopic visualisation was performed following staining with Kwik Diff (Thermo Fischer Scientific).

Flow Cytometry

Tissue of draining popliteal LN or footpad lesions was incubated with Collegenase D (1mg/ml, Roche) and DNASe 1 (100U/ml, Sigma-Aldrich), for 30 minutes at 37°C, disrupted by mechanical disruption between frosted glass slides and single cell suspensions prepared. Cells were filtered through 60μm nylon mesh or 40μm cell strainers (BD Biosciences) to remove tissue debris. Prior to cell surface staining cells were pre-incubated with anti CD16/32 (clone 2.4G2) to block Fc receptors where appropriate. Cells were stained with rat anti-mouse antibodies against B220 (RA3-6B2, Pacific Blue or APC-Cy7), CD11b (M1/70, PerCP-Cy5.5), CD11c (HL3, FITC or PE-Cy7), CD86 (GL1, PE), Ly6G (IA8, PE), CD90.2 (53-2.1, APC), CD4 (RM4-5, PerCP-Cy5.5 or Pacific Blue), CD8 (53-6.7;Pacific Blue) IFN-γ (XMG1.2, Alexa Fluor-488), IL-10 (JES5-16E3, PE), mouse anti-mouse MHC class II ($I\text{-}A^b$; AF6-120.1, biotinylated), CD45.1 (A20, PE), CD45.2 (104, FITC), Armenian-hamster anti-mouse TCR ß-chain (H57-597, APC), CD3

(145-2C11, PE-Cy7) and or polyclonal rabbit anti-mouse iNOS (BD Biosciences). Additionally, rat anti-mouse CD115 (AFS98, Streptavidin APC-Cy7) and Ly6C (ER-MP20, FITC) were purchased from eBiosciences. Annexin V and Streptavadin Pacific Orange were purchased from Invitrogen. Rat anti-mouse CCR2 (MC21) was isolated and purified in the lab of Prof. Matthias Mack (University Hospital, Regensburg, Germany). Unlabeled primary antibodies were detected using either donkey anti-rat IgG Dylight 649 (Jackson ImmunoResearch Laboratories) or goat anti-rabbit IgG Pacific Blue (Invitrogen). Multicolour staining of single cells for surface antigens was performed essentially as published [112]. Data were acquired either using a Cyan ADP (Beckman Coulter) an Aria II (BD Biosciences) or an LSR Fortessa (BD Biosciences). Analysis's was performed using FloJo version 8.86 (Tree Star Inc.).

Purification of murine dendritic cells and monocytes

$CD11c^+$, $CD8^-$ cDC were isolated from spleens and inflammatory monocytes were isolated from bone marrow from femurs of uninfected mice as described previously with minor modifications [320]. Briefly, spleens were minced and were digested with 1mg/mL collegenase D (Roche) in HBSS containing $Mg2^+$ and $Ca2^+$ (Invitrogen) at 37°C prior to passing through 60μM mesh to produce a single cell suspension. Cells were washed in PBS/0.1%BSA 2mM EDTA. Similarly monocytes were isolated from bone marrow obtained from femurs of uninfected mice by flushing with PBS/0.1%BSA using a 26G syringe. Red blood cells from both spleens and bone marrow were lysed in 0.17M ammonium chloride/20mM HEPES buffer. Both splenic and bone marrow cells were separately layered on a 17.2%w/v Histodenz (Sigma Aldritch) in RPMI-1680 (Invitrogen)

gradient and cells at the interface were subsequently sorted using a FACS ARIA II based upon cellular phenotype. Splenic DC were $CD11c^+$, $CD11b^-$, $CD8^-$ and bone marrow inflammatory monocytes were $CD11b^+$, $B220^-$, $Ly6C^+$, $Ly6G^-$. Sorting purity was consistently >98%.

For purification of cellular infiltrate during the course of *L. major* infection, draining pLN were pooled from groups of infected mice at day 21 post infection. Infiltrating myeloid cells were purified as described above from bone marrow monocytes.

Cytokine Analysis.

Cell culture supernatants were collected from monocytic cells either infected *in vitro* with *L. major* promastigotes or stimulated with 1μg/mL LPS or from purified myeloid cells *ex situ* cultured for 24 hours cells from *L. major* infected mice. Cytokine concentrations were measured using mouse inflammation cytokine bead array (CBA, BD Biosciences). CBA were acquired on an ARIA II or LSR Fortessa (BD Biosciences) and FCS 2.0 files were analyzed using FCAP Array (Soft Flow Inc).

Confocal Microscopy

Draining popliteal LNs were dissected and rapidly frozen in Tissue Tek optimal cutting temperature media (OCT) (Pro-Sci-Tech, Townsville, Australia) in liquid nitrogen vapor and stored at -80°C. Sections of 10μm were cut using a cryotome (ThermoShandon), air-dried and fixed in acetone at -20°C. Prior to staining, sections were re-hydrated in PBS 1%

BSA for 60 minutes followed by FC blocking with anti CD16/CD32 (BD Biosciences). Polyclonal IgG antibodies against *L. major* (clone; V121, MHOM/IL/67/Jericho II) were purified from rabbit serum (a kind gift from Emanuela Handman, Walter and Elisa Hall Institute) using Protein G-Sepharose 4B (Invitrogen), followed by labeling with Cy5 mono-reactive dye (Amersham Biosciences, Buckinghamshire, England) as described by the manufacturer.

Staining of pLN sections was performed using polyclonal rabbit anti *L. major* Cy5, mouse anti-mouse iNOS-FITC (6/iNOS/NOS type II; BD Biosciences), and rat anti-mouse CD11b biotin (M1/70; BD Biosciences). Secondary staining was performed using Streptavidin-Alexa 546 (Invitrogen) before being mounted with Mowiol (Calbiochem, La Jolla, CA) containing 2.5% DABCO (Sigma-Aldrich, Australia) to prevent fading and allowed to dry overnight. Sequential images were acquired using a Zeiss LSM710 Confocal microscope in channel mode.

Statistics

Statistical analysis was performed using a non-parametric Mann Whitney test or a Kruskall-Wallace with Dunn's multiple comparison test to test for multiples hypotheses where appropriate. Results are presented as mean values ± SEM. Analyses were performed using GraphPad Prism 5.0 for MacIntosh (GraphPad Software, San Diego California USA, www.graphpad.com). Each experimental group was compared to B6.WT controls. Statistical p values at the level of $p \leq 0.05$ were considered to be significant and were labelled with * $p \leq 0.05$ and ** $p \leq 0.01$, *** $p \leq 0.001$ respectively.

Results

CD11b$^+$ CCR2$^+$ inflammatory monocytes are a major source of TNF ex vivo.

The source of TNF required to promote immunity in murine leishmaniasis has been shown previously to be of haematopoietic origin [112]. To further explore the innate contribution of TNF to a coordinated immune response, I examined the role of Mo-DC that have been shown to comprise a large part of the inflammatory infiltrate in resistant mice [311].

Mo-DC's have been reported to produce large amounts of TNF and have been referred to as TNF/iNOS producing dendritic cells as observed in *Listeria* monocytogenes infection [315]. B6.WT inflammatory Mo-DC purified from bone marrow produce *in vitro* greater quantities of soluble TNF than CD11c$^+$ CD8$^-$ cDC isolated from spleen (Figure 5.1A). Since Mo-DC are capable of producing large quantities of TNF I examined how these cells are involved in the control of *L. major*. Infection of purified B6.WT and B6.TNF$^{-/-}$ bone marrow CCR2$^+$ Mo-DC with *L. major* promastigotes *in vitro* in the absence of stimulating cytokines resulted in clear infection and replication of parasites (See Figure 5.1B). To test if the absence of TNF altered their phenotypic cytokine profile I stimulated purified CD11b$^+$, CCR2$^+$, Ly6C$^+$ Bone marrow Mo-DC and splenic CD11c$^+$, CD8$^-$ cDC with LPS and measured cytokine profile after 24 hours. Both B6.WT and B6.TNF$^{-/-}$ Mo-DC produced greater quantities of IL-6, IL-10 and MCP-1 than CD11c$^+$ CD8$^-$ cDC. There was no significant difference between the genotypes (Figure 5.1C).

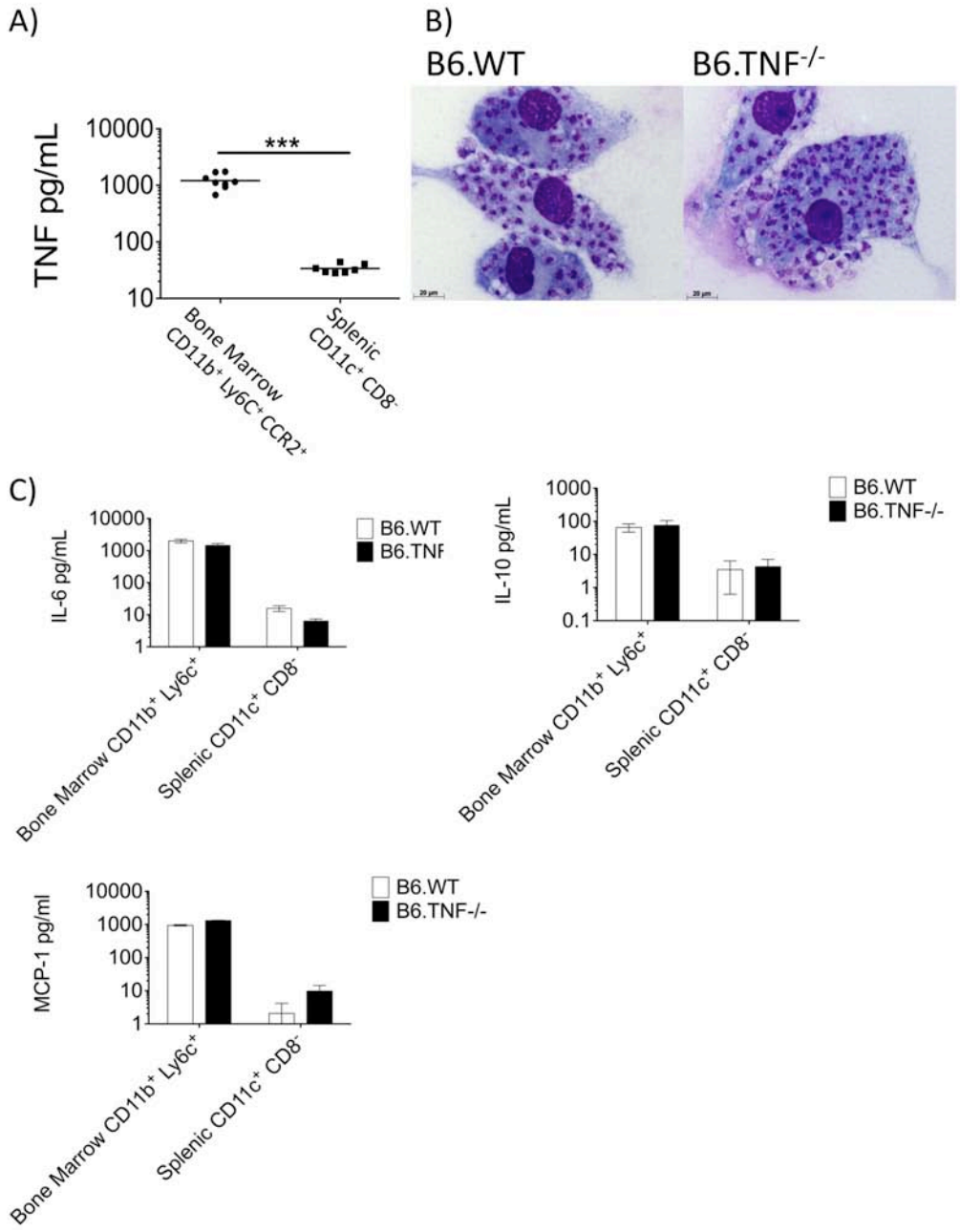


Figure 5.1 CCR2$^+$ Mo- DC are potent producers of TNF and can harbor *L. major*.
A) CCR2$^+$ Mo-DC and CD11c$^+$ cDC were sorted from the bone marrow and spleen of B6.WT mice and stimulated with 1ug/mL LPS. Cell culture supernatant were harvested after 24 hrs for cytokine anslysis n= 6-7, *** =P≤ 0.001, Mann Whitney U test. B) CCR2$^+$ Mo-DC isolated from the bone marrow of B6.WT and B6.TNF$^{-/-}$ mice were purified and infected with *L. major* promastigotes in Nunc Chamber slides. Media was changed daily and after cells were fixed and stained after 72 hrs for examination of intracellular amastigotes. C) Bone marrow CCR2$^+$ Mo-DC and splenic CD11c$^+$ CD8$^-$ cDC were purified from both B6.WT (n=7 and 6, respectively) and B6.TNF$^{-/-}$ (n=3 and 6, respectively) mice stimulated for 24 hours with LPS. Supernatant was harvested and examined for the production of IL-6, IL-10 and MCP-1 by cytometric bead array. Statistical significance was tested using a Mann Whitney U Test.

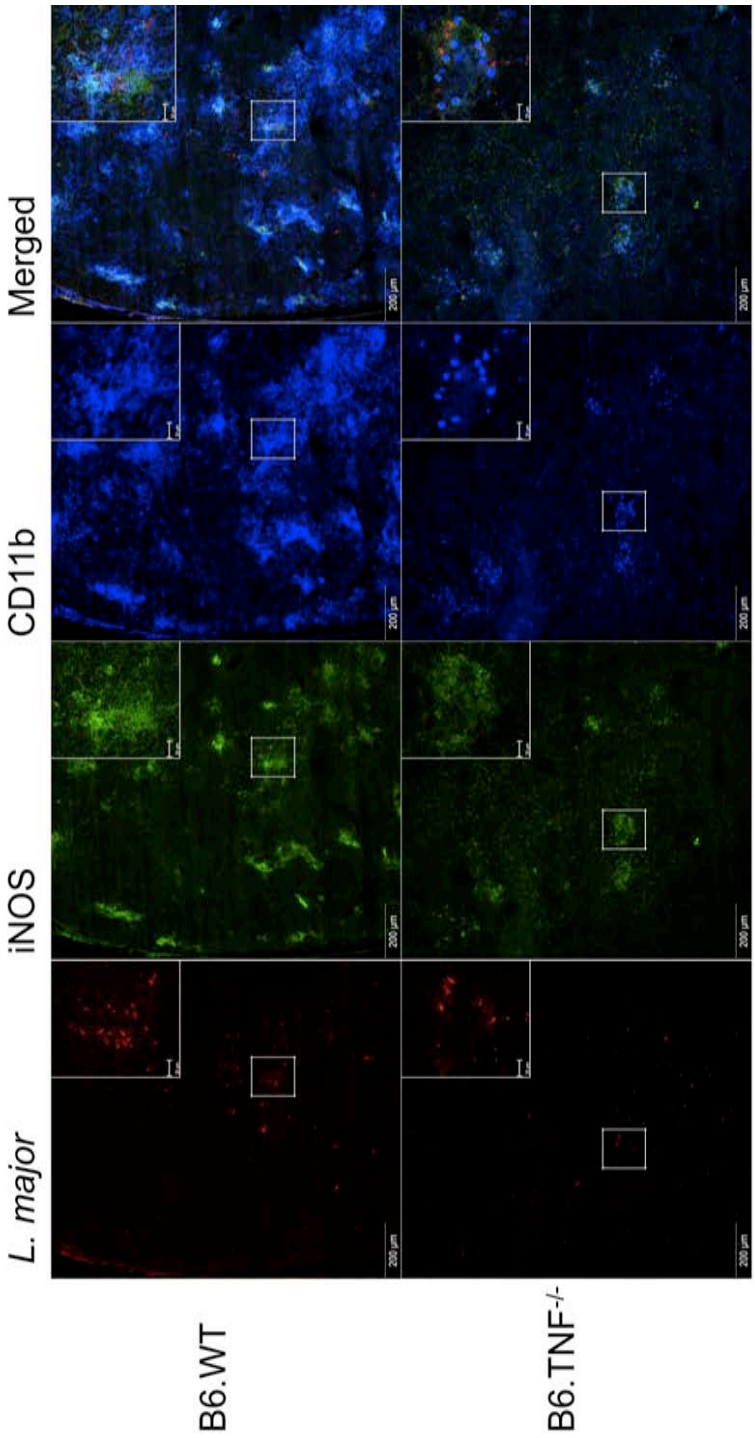


Figure 5.2 Clustering of CD11b^{+} cell with *L. major* parasites in the draining pLN is strongly associated with expression of iNOS.

The localisation of *L. major* parasites was measured with respect the expression of both CD11b and iNOS in B6.WT and B6.TNF-deficient mice in the draining pLN at day 21 post infection. Single channel confocal images of sections stained with polyclonal anti *L. major* IgG (Red), iNOS (Green) and CD11b (Blue) are shown as well as a merged image at10x magnification. Inset shows enlarged view of iNOS clustered regions identified in the image. Scale bar is equivalent to 200μm in the overal image and 20μm in the inset.

Presence of CD11b$^+$ Ly6C$^+$ Mo-DCs is not impaired in L. major infected B6.TNF$^{-/-}$ mice

Since TNF has been shown to influence the early migration of DC from the site of infection to the draining LN [321] I characterised the kinetics and composition of the inflammatory infiltrate during infection with *L. major*. First, I examined *ex situ* the localisation of *L. major* parasites within the draining pLN and the induction of the leishmanicidal enzyme iNOS. In agreement with my previous results (Figure 3.5), both B6.WT and B6.TNF$^{-/-}$ mice showed clustered iNOS$^+$ areas within the pLN at day 21 post infection (Figure 5.2). Previously, this iNOS was predominantly observed within T cell zone (Figure 3.5), and not suprisingly, expression of iNOS was closely paralelled with the presence of *L. major* amastigotes. The expression of iNOS within the draining pLN, similarly, was strongly associated with CD11b$^+$ cells in both B6.WT and B6.TNF$^{-/-}$ mice. Interestingly, while B6.WT show broad clustered patterns of CD11b expression, in the absence of TNF this CD11b expression appears to be more diffuse with the exception of discreet cluters of large CD11b$^+$ cells, that were strongly colocalised to areas of iNOS production and which were absent in B6.WT mice (Figure 5.2).

To further understand how the absence of TNF contributed to changes in the inflamamtory infiltrate in the draining pLN, I undertook a comprehensive flow cytometric analysis of the pLN throughout the course of infection. The analysis of major cDC subsets (CD11c$^+$, CD8; CD11c$^+$, CD8$^+$) and pDC (CD11c$^+$ CD11b$^-$ Ly6C$^+$ B220$^+$) did not reveal substantial differences between B6.WT and B6.TNF$^{-/-}$ over a period of observation of 4 weeks (data not shown). An investigation of inflammatory Mo-DC, which are strong producers of iNOS (CD11c^{low}, CD11b$^{+,}$ Ly6C$^+$, CCR2$^+$) [322], confirmed a strong increase in the size of this population during the course of experimental cutaneous leishmaniasis in B6.WT

mice as described [311] (Figure 5.3A). In the absence of TNF, $CD11b^{+}$, $Ly6C^{+}$, $CCR2^{+}$ Mo-DC were recruited to both the footpad lesion (data not shown) and the draining LN (Figure 5.3A) with statistically increased absolute numbers observed in the draining popliteal LN at the peak of acute disease at day 21 post infection (Figure 5.3B). However, testing for multiple-hypotheses for all four genotypes shows only a statistical difference between B6.WT and B6.TNFR1$^{-/-}$ mice. In contrast, the absence of either TNF or TNFR1 resulted in a significant increase of the total numbers of a $CD11b^{+}$ $Ly6C^{low}$ $CCR2^{low}$ population (Figure 5.3A and B).

Phenotypical characterization of $CD11b^{+}$, $Ly6C^{+}$, $CCR2^{+}$ Mo-DC and $CD11b^{+}$, $Ly6C^{low}$ $CCR2^{low}$ cell populations

To further characterise the infiltrating $CD11b^{+}$ monocyte populations during murine Leishmaniasis Icharacterised the phenotype of both the $CD11b^{+}$, $CCR2^{+}$, $Ly6C^{+}$ Mo-DC population that was observed in both B6.WT, B6.TNF and B6.TNFR-deficient mice as well as a $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ population that accumulated only in TNF and TNFR1-deficient mice (Figure 5.4A). The $CD11b^{+}$, $CCR2^{+}$, $Ly6C^{+}$ Mo-DC population was phenotypically similar to that already described for inflammatory monocytes, having a typical leukocyte morphology as represented in their forward and side scatter distributions. Both B6.WT and B6.TNFR-deficient Mo-DC expressed high levels of MHC II and CD86 with B6.TNF$^{-/-}$ and B6.TNFR1$^{-/-}$ displaying slightly higher levels than either B6.WT or B6.TNFR2$^{-/-}$ (Figure 5.4 B) indicating that a large proportion of these cells were activated and capable of stimulating T cell responses.

In contrast, the accumulating $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells observed in B6.$TNF^{-/-}$ and B6.$TNFR1^{-/-}$ were predominantly negative for MHC II and the activation marker CD86 in all genotypes. While, $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells from B6.WT and B6.$TNFR2^{-/-}$ cells displayed a characteristically small morphology as determined by forward and side scatter both B6.$TNF^{-/-}$ and B6.$TNFR1^{-/-}$ which showed the greatest accumulation of this cell type had greatly increased side scatter profiles (See Figure 5.4 C) indicating a more granular morphology.

Further phenotypic analysis showed that the $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells that appeared in B6.WT and B6.$TNFR2^{-/-}$ mice were fundamentally different from the $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells that accumulate in B6.$TNF^{-/-}$ and B6.$TNFR1^{-/-}$ mice. $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ from B6.WT mice cells expressed increased levels of Annexin V ($p \leq 0.001$ and $p \leq 0.05$ respectively) as well as reduced levels of CD115, which did not reach statistical significance when multiple hypothesis comparison was implemented. In addition, a proportion of these cells observed in B6.WT and B6.$TNFR2^{-/-}$ mice expressed high levels of Ly6G indicating that these cells might be granulocytes such as neutrophils (Figure 5.4 lower panel).

Conversely, B6.$TNF^{-/-}$ and B6.$TNFR1^{-/-}$ $CD11b^{+}$, $CCR2^{+}$, $Ly6C^{+}$ Mo-DC showed a similar phenotype irrespective of the genotype with the exception of an increase in expression of Annexin V in B6.$TNF^{-/-}$ and B6.$TNFR1^{-/-}$ mice (Figure 5.4 D and E).

At day 21 after infection, both B6.WT and B6.$TNF^{-/-}$ $CD11b^{+}$, $CCR2^{+}$, $Ly6C^{+}$ Mo-DC from draining pLN resemble monocytes histologically and produce both IL-6 and IL-10 which is reduced significantly in B6.$TNF^{-/-}$ mice (Figure 5.5). In contrast, $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells are histologically distinct between B6.WT and B6.$TNF^{-/-}$, with

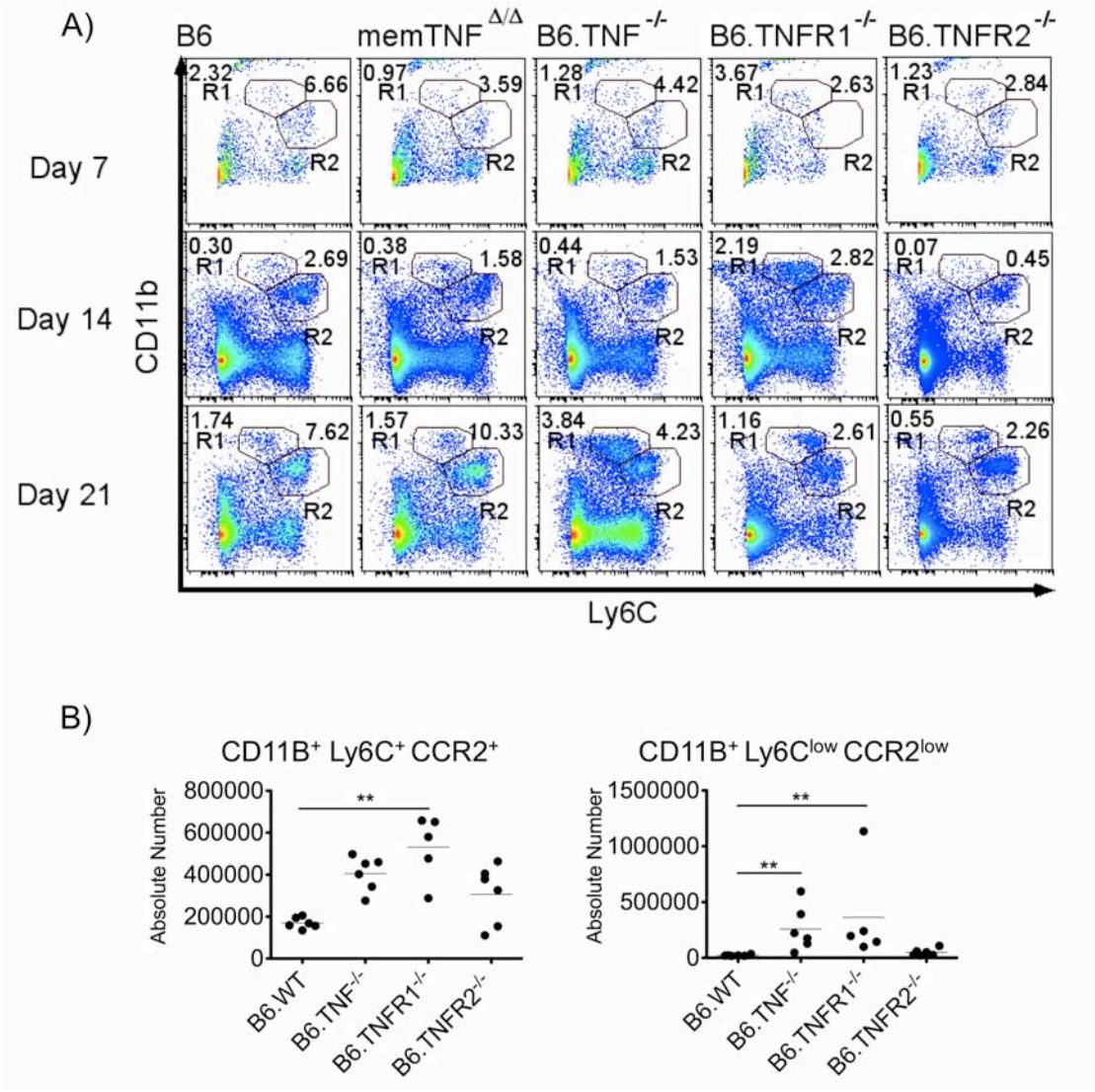


Figure 5.3 Comparison of the inflammatory infiltrate during the course of *L. major* infection in B6.WT, B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$ and B6.TNFR2$^{-/-}$ mice.

(A) CD11b^{+} Ly6C^{low} monocytes (R1) and CD11b^{+} Ly6C^{+} inflammatory Mo-DC (R2) are recruited into draining pLN during the course of *L. major* infection (day seven, day 14 and day 21). A dot plot representative of three experiments (n = 5-6 mice/experiment) is shown. (B) Summary of the absolute numbers of CD11b^{+} Ly6C^{low} CCR2^{low} monocytic cells and inflammatory CD11b^{+} Ly6C^{+} CCR2^{+} Mo-DC of B6.WT and B6.TNF$^{-/-}$ mice from one experiment at day 21 after infection is shown. Every point represents one individual mouse (n = 5-6, ** $p \leq 0.01$, Statisitical significance was tested using Kruskal-Wallis with Dunn's multiple comparison test.)

B6.WT $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ resembling neutrophils, having a distinct polymorphonuclear appearance while $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells from B6.$TNF^{-/-}$ are mononuclear and take on a veiled appearance (Figure 5.5).

The $CD11b^{+}$ $Ly6C^{low}$ $CCR2^{low}$ population harbours L. major and does not produce NO

Previously, I showed that B6.$TNF^{-/-}$ and B6.$TNFR1^{-/-}$ mice develop robust T cell responses, produce large quantities of IFN-γ and express iNOS in both the footpad lesion and draining LN (See Chapter 3). Since both TNF and TNFR1-deficent mice show marked disruptions in the microarchitecture of the secondary lymphoid organs [5, 323] I could not exclude the possibility that the previously observed expression of iNOS was spatially separated from *L. major* infected cells [112]. To investigate this, I utilised a transgenic eGFP expressing *L. major* (MHOM/IL/80/*Friedlin* eGFP) to ascertain the *in vivo* localization of the parasite, together with IFN-γ-dependent effector functions such as NO production in pLN infiltrating Mo-DC.

Examination of the draining pLN at day 21 post infection revealed the accumulation of two distinct $CD11b^{+}$ myeloid populations in B6.$TNF^{-/-}$ mice that differed with respect to both iNOS expression and the presence of $eGFP^{+}$ *L. major* amastigotes and correlated with the two distinct $CD11b^{+}$ myeloid population observed previously in B6.$TNF^{-/-}$ and B6.$TNFR1^{-/-}$ infected mice (Figure 5.3 and 5.4). The population in R1 corresponded to the $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ population while R2 was the equivalent of $CD11b^{+}$, $CCR2^{+}$, $Ly6C^{+}$ Mo-DC described in Figure 5.3 and 5.4. In $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ population (R1), the size of the uninfected population remained largely unchanged in both genotypes, while in B6.$TNF^{-/-}$ mice, the *L. major* harbouring cell population had become dominant (Figure 5.6).

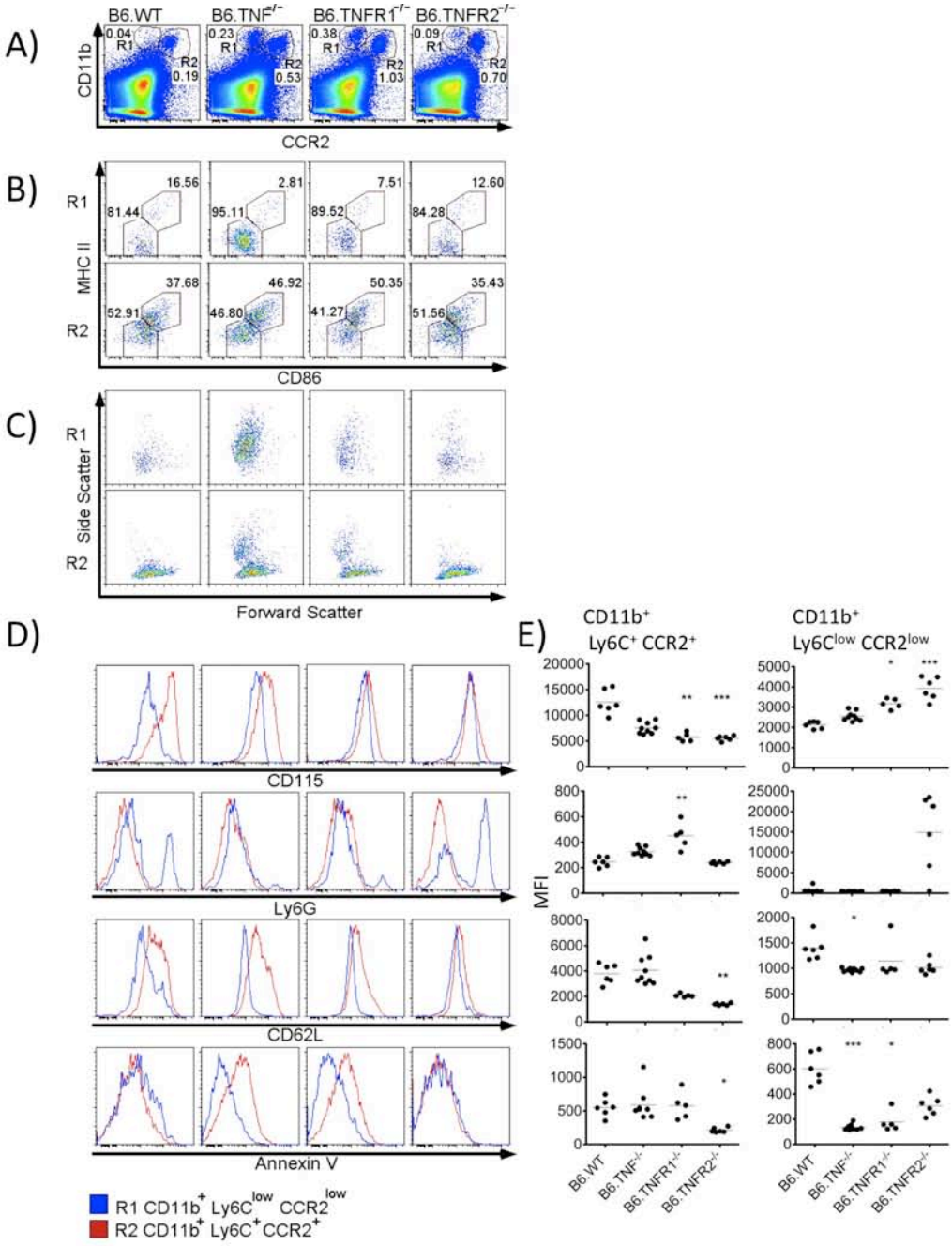


Figure 5.4 Phenotypic characterization of CD11b^{+}, Ly6C^{low}, CCR2^{low} monocytic cells and inflammatory CD11b^{+}, Ly6C^{+}, CCR2^{+} Mo-DC of B6.WT, B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$ and B6.TNFR2$^{-/-}$ mice.

A representative dot plot characterizing the infiltrating myeloid cells in B6.WT, B6.memTNF$^{\Delta/\Delta}$, B6.TNF$^{-/-}$, B6.TNFR1$^{-/-}$, B6.TNFR2$^{-/-}$ mice is shown in (A). The CD11b^{+} Ly6C^{low} CCR2^{low} monocytic cells (R1) and the inflammatory CD11b^{+} Ly6C^{+} CCR2^{+} Mo-DC (R2) are analysed regarding their activation (MHC class II and CD86, (B)), their scatter morphology (FSC/SSC, (C)) and their expression of CD115, Ly6G, CD62L and Annexin V (D) Median fluorescent intensity is shown in (E) for all four genotypes. (n= 6-7 per genotype * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ Statistical significance was tested using Kruskal-Wallis with Dunn's multiple comparison test.)

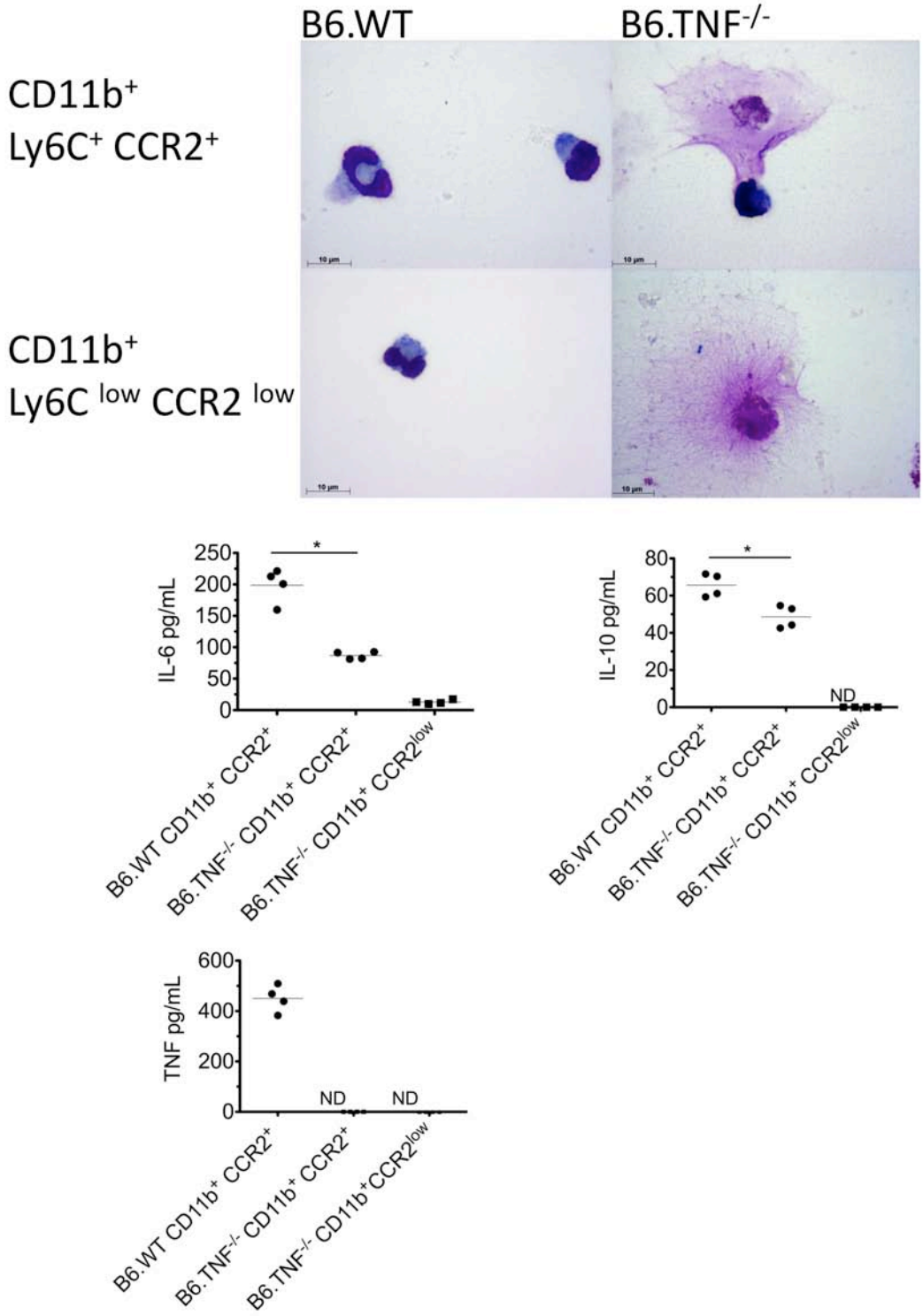


Figure 5.5 Histological morphology of CD11b$^+$ CCR2$^+$ Ly6C$^+$ and CD11b$^+$ CCR2^{low} Ly6C^{low} cells from *L. major* infected B6.WT and B6.TNF$^{-/-}$ mice.

B6.WT and B6.TNF-/- were infected with 3x10^6 *L. major* promastigotes and draining pLN were harvested at day 21 post infection. CD11b$^+$ CCR2$^+$ Ly6C$^+$ and CD11b$^+$ CCR2^{low} purified by flow cytometry. 4x10^5 cells were incubated for 24 hours *ex vivo* and supernatants harvested for analysis of cytokines using mouse inflammation cytometric bead array (BD Biosciences). * $p \leq 0.05$, ND= Not detected.

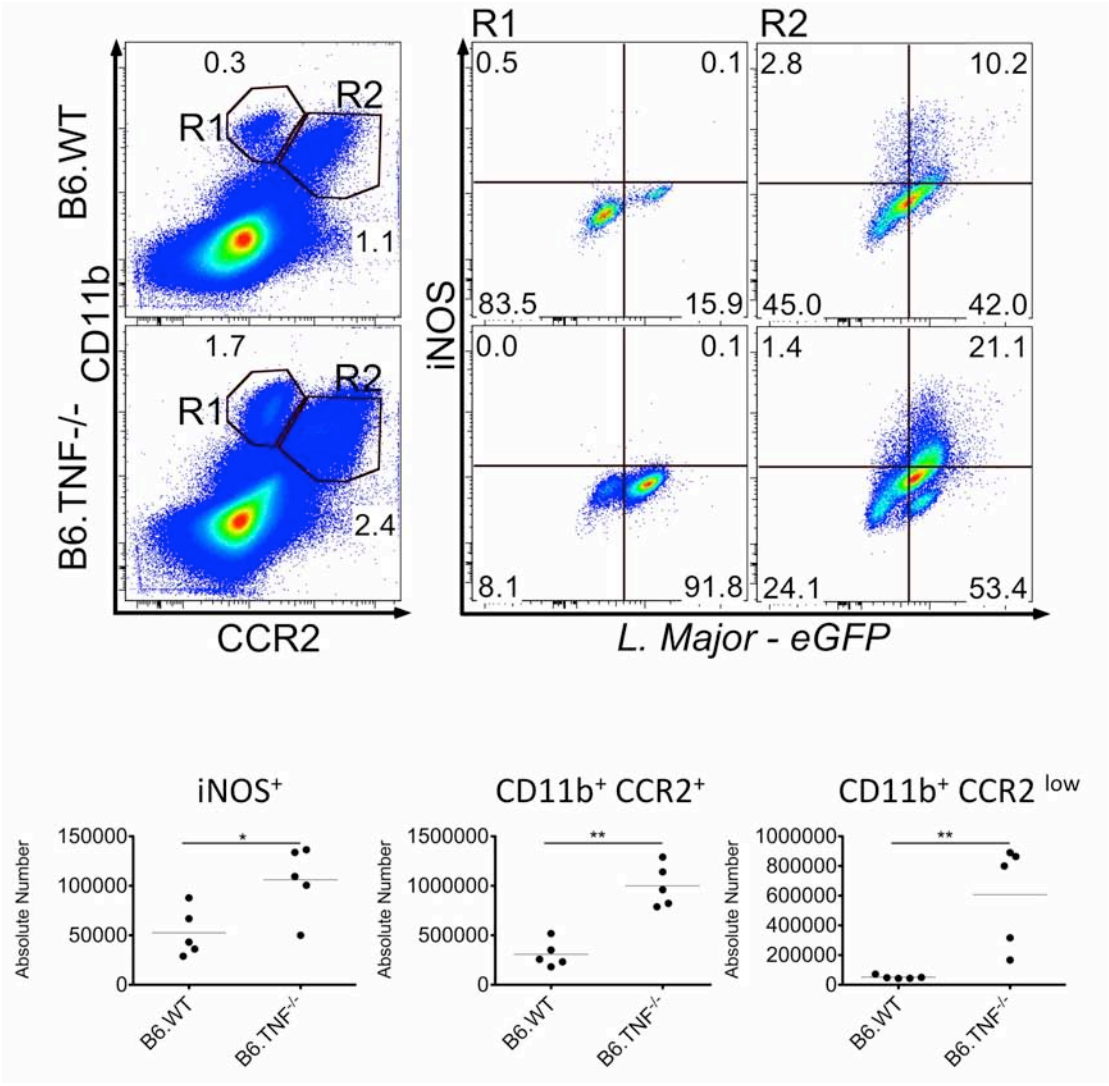


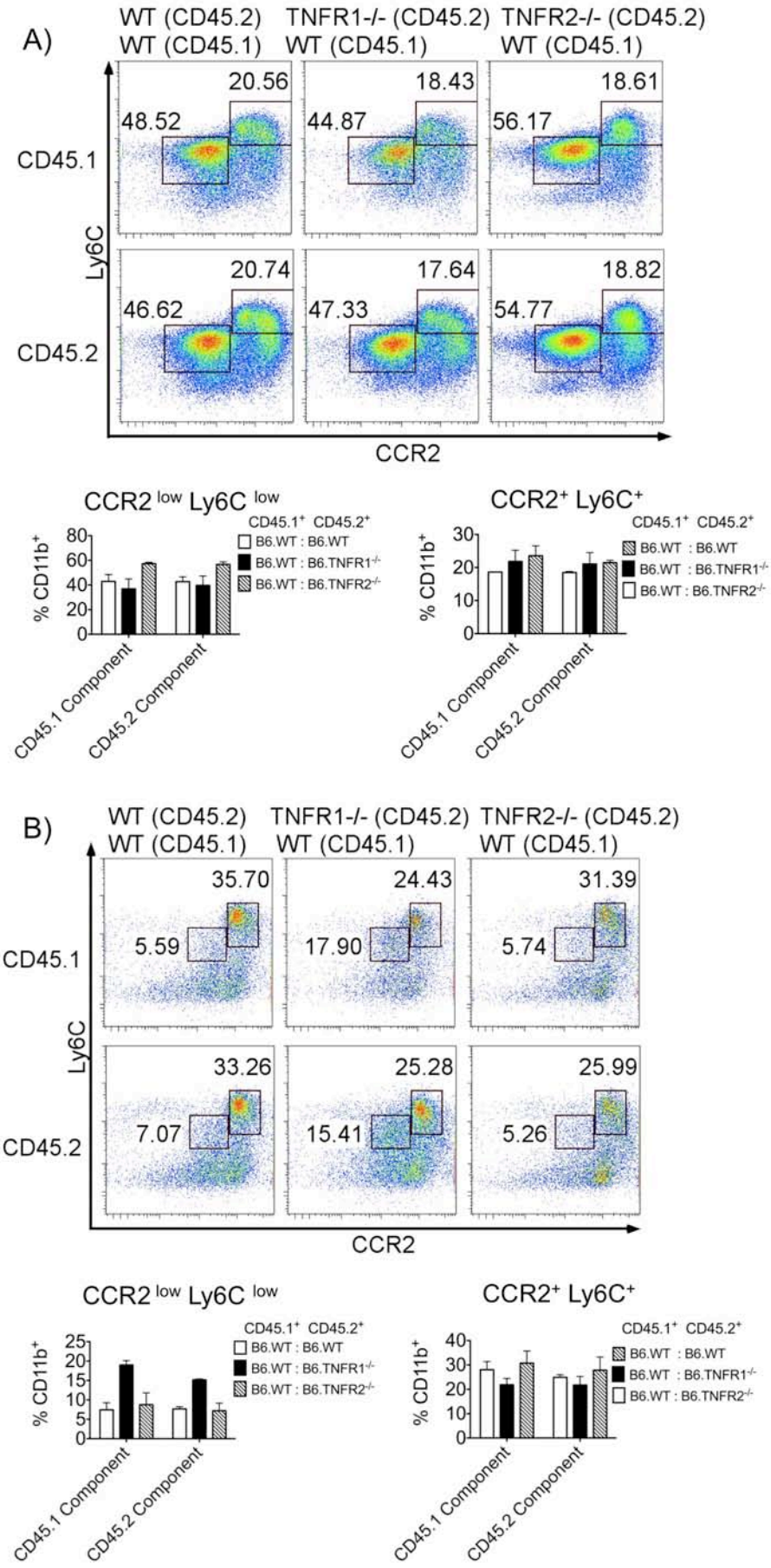
Figure 5.6 Characterization of iNOS expression in CD11b^{+} CCR2^{+} and CD11b^{+} CCR2^{low} cells from B6.WT and B6.TNF$^{-/-}$ mice infected with fluorescent eGFP-*L. major*.

B6.WT and B6.TNF$^{-/-}$ mice were infected with eGFP-*L.major* promastigotes and draining pLN were taken at day 21 after infection. CD11b^{+} CCR2^{low} Ly6C^{low} monocytic cells (R1) and CD11b^{+} CCR2^{+}Ly6C^{+} Mo-DC (R2) were analysed by flow cytometry.for intracellular iNOS and eGFP expression. The absolute numbers of total iNOS^{+} cells and of CD11b^{+} CCR2^{+} and CD11b^{+} CCR2^{low} inflammatory cells were also calculated (n=5). * $p \leq 0.05$, ** $p \leq 0.01$, Statisitical significance was tested using Mann Whitney U test. One of two independent experiments is shown.

Conversely, $CD11b^+$, $CCR2^+$, $Ly6C^+$ Mo-DC (R2, Figure 5.6) from both B6.WT and $B6.TNF^{-/-}$ mice displayed a substantial parasitic burden of *L.major*-eGFP but showed concurrent iNOS expression (Figure 5.6). Quantitative analysis of the pLN revealed an overall increase in total number of $iNOS^+$ cells in the pLN of $B6.TNF^{-/-}$ mice that corresponded with an overall increase in the numbers of $CD11b^+$ $CCR2^+$ $Ly6C^+$ Mo-DC.

Development of infected myeloid cells is not dependant on intrinsic responsiveness to TNF signalling.

Since TNF has been shown to influence dendritic cell and MΦ development and activation, changes in monocytic developmental due to the absence of TNF or its receptors may influence the establishment of an appropriate immune response. To test how intrinsic responsiveness to TNF is correlated with susceptibility I generated mixed bone marrow chimeras utilising B6.WT, B6.TNFR1 and B6.TNFR2-deficient bone marrow in a three-way congenic set up which allowed me to examine the individual contribution of TNFR1 or TNFR2 signalling to the development of the monocytic infiltrate. The genetic identity of individual myeloid cells was analysed in footpad and draining LN at day 14 after infection of the chimeric mice with *L. major* promastigotes. Analysis of the footpad lesion showed remarkable similarities between all three mixed chimeras with strong influx of both $CD11b^+$, $CCR2^+$, $Ly6C^+$ and $CD11b^+$, $CCR2^{low}$, $Ly6C^{low}$ cells (Figure 5.7A). However, the $CCR2^{low}$, $Ly6C^{low}$ myeloid cells observed in the footpad expressed higher levels of Ly6G that their LN counterparts identified previously and were more akin to neutrophilic than monocytic infiltrate (data not shown). In the draining popliteal LN the donor combination of WT/WT and WT/TNFR2 developed a strong $CD11b^+$, $CCR2^+$


A)
WT (CD45.2)
WT (CD45.1)
TNFR1-/- (CD45.2)
WT (CD45.1)
TNFR2-/- (CD45.2)
WT (CD45.1)
20.56
18.43
18.61
48.52
44.87
56.17
20.74
17.64
18.82
46.62
47.33
54.77
CD45.1
CD45.2
Ly6C
CCR2
CCR2 low Ly6C low
CCR2+ Ly6C+
CD45.1+ CD45.2+
B6.WT : B6.WT
B6.WT : B6.TNFR1-/-
B6.WT : B6.TNFR2-/-
% CD11b+
CD45.1 Component
CD45.2 Component
B)
WT (CD45.2)
WT (CD45.1)
TNFR1-/- (CD45.2)
WT (CD45.1)
TNFR2-/- (CD45.2)
WT (CD45.1)
35.70
24.43
31.39
5.59
17.90
5.74
33.26
25.28
25.99
7.07
15.41
5.26
CD45.1
CD45.2
Ly6C
CCR2
CCR2 low Ly6C low
CCR2+ Ly6C+
CD45.1+ CD45.2+
B6.WT : B6.WT
B6.WT : B6.TNFR1-/-
B6.WT : B6.TNFR2-/-
% CD11b+
CD45.1 Component
CD45.2 Component

inflammatory Mo-DC population, which was derived equally from both donor genotypes. The $CD11b^{+}$, $Ly6C^{low}$, $CCR2^{low}$ cell population previously described (R1, Figure 5.3, 5.4 and 5.6), which had been shown to maintain a high parasite load was hardly detectable (Figure 5.7B). In contrast, the WT/TNFR1 mixed chimera developed strong populations of both $CD11b^{+}$, $CCR2^{+}$, $Ly6C^{+}$ inflammatory Mo-DC and $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells (Figure 5.7 B). Interestingly, in this experimental set up both these cell populations could be shown to originate equally from both WT and $TNFR1^{-/-}$ bone marrow progenitor cells as indicated by their congenic label. The relative increase in $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells derived from both WT and TNFR1-deficient donor cells was also paralleled by a small-observed decrease in the proportions of $CD11b^{+}$, $CCR2^{+}$, $Ly6C^{+}$ inflammatory Mo-DC that did not reach statistical significance (Figure 5.7) indicating that these two populations may reflect a developmental continuum.

Figure 5.7 Intrinsic responsiveness to TNF is not sufficient for the development of a resistant monocyte phenotype during infection with *L. major*.

Mixed bone marrow chimeras containing either WT (CD45.1)/WT (CD45.2), WT (CD45.1)/$TNFR1^{-/-}$ (CD45.2), WT (CD45.1)/$TNFR2^{-/-}$ (CD45.2) bone marrow were infected with *L. major* promastigotes six weeks following bone marrow reconstitution. Footpad lesions and draining pLN were harvested 14 days post infection and the composition of the inflammatory infiltrate analysed by flow cytometry based on allelic differences in CD45. Proportion of WT derived (CD45.1) and WT, $TNR1^{-/-}$ or $TNFR2^{-/-}$ (CD45.2) derived $CD11b^{+}$ $Ly6C^{low}$ $CCR2^{low}$ and $CD11b^{+}$ $Ly6c^{+}$ $CCR2^{+}$ infiltrating cells in the footpad (A) or the draining pLN (B) of *L. major* infected mixed bone marrow chimeras. n=3 mice per combination.

Discussion

A role for TNF and its receptors in mediating resistance to intracellular infection, such as experimental cutaneous leishmaniasis, is well established [106, 107, 112]. In the absence of soluble TNF or TNFR1 the response to *L. major* is severely impaired and results in progressive and maintained infection that coincides with visceralization of the parasite and ultimately leads to death [112]. The mechanisms that are relevant for the lack of resistance in the absence of TNF are still not understood. Previously, it has been shown that TNF or TNFR1-deficient mice develop exacerbated cellular immune responses characterised by increased IFN-γ production upon infection with *L. major* or *Mycobacteria tuberculosis* [324-326]. However, despite the development of the normally protective IFN-γ response, TNF-negative mice infected with *L. major* still fail to resolve cutaneous lesions and succumb to infection despite the resistant genetic background [68, 271, 272]. In this model, the TNF necessary to control susceptibility to *L. major* infection in resistant C57BL/6 mice is generated by cells of haematopoietic origin [112].

In the *L. monocytogenes* infection model, the TNF expression in neutrophils and macrophages was abolished using a targeted deletion of TNF under the lysozyme M promoter. The infection of these modified mice resulted in a rapidly fatal outcome comparable to the infection of TNF-deficient mice demonstrating the importance of TNF produced by these cells [327]. Furthermore, the membrane form of TNF promoted resistance to infection in both *L. major* infection [113] (PDF and HK, unpublished data) and in *M. tuberculosis* infection. In this model a membrane TNF expression by non lymphoid haematopoietic cells was emphasized in particular [325]. In our study, I focused on the monocytic response to *L. major* in B6.TNF$^{-/-}$ mice and present data describing the

accumulation of $CCR2^{+}$, $Ly6C^{+}$ Mo-DC and an yet unknown $CCR2^{low}$, $Ly6C^{low}$ monocytic cell population at the peak of disease in TNF or TNFR1-deficient mice and present *in vivo* evidence for a role for reverse signalling through membrane TNF.

One of the major effector mechanism employed by the immune system in dealing with intracellular pathogens is the induction of iNOS in cells of the mononuclear phagocyte system. The presence of NO in this manner has been shown to be vitally important in the control of *L. major in vivo* [244, 257] and *in vitro* [328-330]. TNF has been shown *in vitro* to be a potent co-activator of macrophages for the production of NO in leishmaniasis and in other infections [106, 227, 229, 306-309]. Recently, a cellular source of NO has been identified *in vivo* that has been ascribed to infiltrating myeloid cells observed in a number of intracellular infections. These cells expressed both iNOS and TNF and have been referred to as TIP-DC, inflammatory monocytes or Mo-DC [331]. Infection with intracellular parasites such as *L. monocytogenes or T. gondii* results in accumulation of $CCR2^{+}$ Tip-DC in the spleens or peritoneum of infected mice, which produce iNOS and TNF, colocalise with the intracellular pathogens and are critical for the resolution of the infection [315, 317, 318, 332, 333]. Similarly infection with *L. major* results in strong recruitment of TNF/iNOS producing Mo-DC to the lesion and draining LN [311, 312] which is important for the initiation of protective anti-parasitic responses [319]. Initial characterisation of Mo-DC in the context of *L. major* infection showed that dermal and LN Mo-DC differed in their expression of Ly6C and increased MHC II and CD86 in dermal derived Mo-DC [311] as compared to LN Mo-DC, which was described as the consequence of a maturation of these cells on their way from the site of infection to the lymphoid organ. In my model of *L. major* infection, I have shown a similar recruitment of Mo-DC to the footpad lesion and subsequently to the draining LN without the described

downregulation of Ly6C and CCR2 in B6.WT mice. In contrast to *L. major* infection in other models, both TNF and TNFR1 deficient mice show an accentuated accumulation of $CCR2^{low}$, $Ly6C^{low}$ cells monocytes in the LN. Phenotypically these cells resemble alternatively activated MΦ (M2) or a subset of myeloid derived suppressor cells. Both cell populations have been characterised by their low expression of CCR2, Ly6C and CD62L and have been shown to exert suppressive effects, notably through modulating T cell functions [334, 335]. While in the results presented, the $CCR2^{low}$ $Ly6C^{low}$ cells are characteristically small, their granular morphology observed during infection with *L. major* is most likely accounted for by the number of intracellular *L. major* amastigotes harboured, which indicates a role for TNF in promoting induction of iNOS in these cells but not in Mo-DC. Alternatively, the accumulation of these infected cells may reflect an accumulation of maturing Mo-DC that down regulate CCR2 and Ly6C during maturation as a result of an increased parasitic burden or through suppressive mechanisms induced by the parasite that can normally be overcome in the presence of TNF.

Previous experiments have shown that TNF deficiency is associated with changes in micro-anatomical organisation of the secondary lymphoid organs[5, 19, 336]. TNF-dependent induction of CCL19 and CCL21 chemokine gradients promotes compartmentalisation of T and B cells [5, 6] and instructs for dendritic cell migration from the periphery to T cell rich areas of the secondary lymphoid organs [337, 338]. In contrast, monocyte recruitment to inflamed sites appears to result from TNF independent mechanisms and relies primarily on ligands for CCR2 but not necessarily on the primary ligand CCL2 for migration to inflamed peripheral tissues [320]. The results generated in my *L. major* infection model are in agreement with this general concept of inflammatory monocyte recruitment to inflamed tissues independent of TNF. At day 21 after infection,

which represents the peak of acute disease, both the number of $CCR2^{+}$, $Ly6C^{+}$ inflammatory monocytes as well as $iNOS^{+}$ cells are present in significantly increased numbers in TNF-deficient mice as compared to infected B6.WT mice. Interestingly, findings reported recently in leishmaniasis show a marked reduction in the numbers of $CD11c^{+}$, $CD11b^{+}$, $iNOS^{+}$ cells in $TNF^{-/-}$ mice of a mixed genetic background at day 28 post infection [312]. This obvious discrepancy could be due to the later time point at which the tissue was analysed or the use of a transgenic *L. major* strain in contrast to the highly infective BNI strain used in my experiments.

Recent experiments suggest that $CCR2^{+,}$ $Ly6C^{+}$, $CXC3R1^{intermediate}$ monocytes can convert into $CCR2^{low}$, $Ly6C^{low}$, $CXC3R1^{high}$ monocytes as they mature in both bone marrow and blood [339, 340]. Whether these $CCR2^{low}$, $Ly6C^{low}$, $CXC3R1^{high}$ monocytes represent the same parasitised $CCR2^{low}$, $Ly6C^{low}$ cells observed accumulating in the absence of TNF or TNFR1 during infection with *L. major* is unclear and difficult to establish. The functional differences between these two monocyte populations are still widely discussed[339-343]. $CCR2^{+}$ $Ly6C^{+}$ monocytes have been shown to give rise to a number of different macrophage and DC population including TipDC that are important in a number of intracellular infections. Steady state $CCR2^{low}$ $Ly6C^{low}$ $CXC3R1^{high}$ monocytes, however, appear to serve a more homeostatic or regulatory function and studies suggest they may perform a surveillance function in post capillary venules [344]. Interestingly, these cells were the first to extravasate into the peritoneum following i.p infection with *L. monocytogenes* and produced an early burst of TNF followed by differentiation into macrophages exhibiting an alternatively activated phenotype. This is similar following myocardial infarction, where $Ly6C^{low}$ monocytes exhibited reduced inflammatory potential and proangiogenic functions [345].

In contrast to these described maturation processes, the accumulation of $CD11b^{+}$, $CCR2^{low}$, $Ly6C^{low}$ cells observed in the absence of TNF and TNFR1 may represent an accumulation of monocytes that derive from the $CD11b^{+}$, $CCR2^{+}$, $Ly6C^{+}$ infiltrating Mo-DC. The reason for the accumulation of these cells and the high degree of parasitism observed in these cells may reflect differential additive defects, such as the impaired induction of anti-parasitic effector molecules such as NO, which may correlate with reduced anti-leishmanicidal capacity, or through impaired migration within the secondary lymphoid organs due to the absence of intact TNFR1 signalling. In addition, the level of Annexin V staining was also decreased in $CD11b^{+}CCR2^{low}$ $Ly6C^{low}$ cells compared to phenotypically equivalent B6.WT cells with intact TNFR1 signalling. This raises the question of whether this apparent decrease in apoptotic potential results from defective TNF signalling or through a parasite host interaction that is normally overcome in the presence of TNF.

Evaluation of WT/TNFR-deficient mixed bone marrow chimeras during infection with *L. major* allowed us to evaluate the specific intrinsic contribution of TNF signalling to the development of the inflammatory monocyte compartment from genetically distinct bone marrow progenitors. The accumulation of parasitised $CCR2^{low}$ $Ly6C^{low}$ myeloid cells from both B6.WT and B6.$TNFR1^{-/-}$ derived bone marrow progenitors would argue for either a parasite/host interaction that renders them susceptible to infection or for TNF acting upstream of intrisic induction of iNOS to promote an effective leishmanicidal innate effector response.

It is tempting to speculate that a functional link exists between the observed phenotypes of both TNF- and CCR2-deficient mice in response to *L. major* [319]. Impaired extravasation

of $CCR2^{+}$ monocytes from the bone marrow or the inability of these cells to produce TNF may render an inescapable block in effective innate immune response to intracellular pathogens. However, since these $CCR2^{low}$ $Ly6C^{low}$ cells, which are associated with a susceptible phenotype in my infection model and arise from both WT and TNFR1-deficient bone marrow progenitors in mixed bone marrow chimeras, the intrinsic role for TNF in controlling their infectivity has to be questioned. This is supported by my data that shows that that memTNF mice do not display this cellular phenotype. A role for membrane TNF on myeloid bone marrow progenitors in conveying protective function to intracellular infection with *M. tuberculosis* has been shown in reciprocal $memTNF^{\Delta/\Delta}/TNF^{-/-}$ bone marrow chimeras in which haemopoietic cells derived from $memTNF^{\Delta/\Delta}$ mice conveyed a protective phenotype to infection while adoptive transfer of either WT or $memTNF^{\Delta/\Delta}$ T cells did not [346]. This is in agreement with earlier observation that cells of haematopoietic origin need to be able to produce TNF to facilitate control of intracellular *L. major* parasites [112].

Taken together my results argue for a role for membrane TNF signalling on infiltrating monocytes with either soluble or membrane bound TNFR1, on a different cellular subtype that promotes either monocyte differentiation or licences the acquisition of leishmanicidal functions in infected cells independently of IFN-γ.

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Chapter 6: General Discussion

The roles for TNF in co-regulating the immune system are multifaceted and complex. Different studies have implicated TNF in contributing to the pathologies associated with a number of human diseases [14] including Rheumatoid arthritis (RA)[347-350], cancer [8-10] and type 2 diabetes mellitus [11, 12]. Consequently, it has become the target of pharmacological interventions and anti-TNF treatments are ,meanwhile, the standard of care in a number of chronic inflammatory conditions such as RA [13], ankylosing spondylitis [351, 352], and inflammatory bowel diseases such as Crohn's disease [115] and ulcerative colitis [353]. However, despite the now widespread use of anti-TNF therapies in disease modulating therapies, the increased recrudescence of latent infections such as tuberculosis [117, 118] and leishmaniasis [119-122, 354] requires increased screening for infectious disease and vigilance on the part of clinicians.

In this thesis, I examined the role that TNF plays in the context of infectious experimental cutaneous leishmaniasis and revealed a role for TNF in regulating the immune response to deal with this intracellular infection. A comprehensive study of the existing literature demonstrated that TNF can exert both protective and pathological effects in different models of parasitic infection (Chapter 2). For example, in cerebral malaria increased TNF observed in patients had been associated with susceptibility, however, neutralisation by means of treatment with anti-TNF mAb did not result in any increased survival, but rather contributed towards increased neurological sequela [184, 355]. Similarly, the contribution of TNF neutralisation appeared to be closely linked with host genetic predisposition; neutralisation of TNF in susceptible mice resulted in decreased mortality to cerebral malaria [158, 194, 195], while similar treatments in resistant mice confered an increase in

severity [157]. In contrast, during trypansomiasis induced by infection with *T. cruzi*, treatment of susceptible BALB/c mice with recombinant TNF resulted in a more acute disease progression, while anti-TNF mAb treatment displayed varying clinical outcomes ranging from increasing cachexia and mortality to a partial resistance and decreased cachexia [233, 234] depending on the clone specificity of the antibody used. These disconcordant outcomes of TNF neutralisation, either through cytokine neutralisation or through genetic disruption, failed to resolve the underlying contribution of TNF to mediating the balance between immune protection and immune mediated pathology.

In the context of experimental cutaneous leishmaniasis, I clearly demonstrated that IFN-γ responses and leishmanicidal activity were dissociated in the absence of TNF, and that membrane TNF and TNFR1 were essential to the development of a protective immune response. Furthermore, I showed that TNF actes on myeloid cells of the innate immune system to regulate differentiation and leishmanicidal potential upstream of the intrinsic ability of TNF to co-stimulate direct induction of NO by iNOS. Similarly, I have described how the absence of TNF affected the $CD4^+$ T cell response following challenge with *L. major* resulting in increased T cell activation and epitope spreading.

The exploration of TNF signalling *in vivo* in response to infection has been hampered by the use of gene deficient models of poorly defined genetic backgrounds resulting from the high degree of linkage associated with the location of the *Tnf* gene, within the MHC. This has potentially contributed to the conflicting observations and interpretations arising from infection studies. In the case of cutaneous leishmaniasis I have sought to address this issue through the use of TNF and TNFR mice that have been generated either directly using B6 ES cells (B6.$TNF^{-/-}$, B6.mem$TNF^{\Delta/\Delta}$ or B6.$TNFR2^{-/-}$) or have been backcrossed greater

than 10 times (B6.TNFR1$^{-/-}$). Using this approach I was able to confirm the importance of TNF in mediating protection to *L. major* (MHOM/IL/81/FE/BNI) but was unable to reproduce the results described previously for TNFR1 and TNFR2, in which TNFR1-deficient mice resolved the infection and survived while showing diminished capacity to heal the lesion, and TNFR2-deficient mice were completely resistant [110]. In contrast, in my model both TNFR1 and TNFR2 appear to share overlapping roles in mediating protection and healing of parasite lesions. However, similar to experiments published previously, TNFR2 does not appear to be essential for the induction of protective immunity as these mice (B6.TNFR2$^{-/-}$) ultimately survive the infection, in contrast to B6.TNFR1-deficent mice, which display a similar disease course to B6.TNF-deficent mice and ultimately, succumb to *L. major*.

The induction of IFN-γ has long been considered to be paramount to protective immunity to infection with intracellular parasites such as *L. major*, due to its effect on inducing effector functions, such as the production of reactive nitrogen species in parasitised cells such as macrophages at sites of infection [106]. A number of reports support a role for TNF in synergising with IFN-γ in inducing NO production through regulating expression of iNOS both *in vivo* [106] and *in vitro* [273, 278-280]. The apparent contribution of TNF to regulating iNOS induction during parasitic infection was highlighted in a number of infection models including malaria infection studies where treatment of *Plasmodium chabaudi* infected mice with either anti-TNF or a combination of anti-TNF and anti-IFN-γ monoclonal antibodies resulted in the reduction of splenic iNOS expression and a corresponding decrease in serum nitrite (NO_3^-). This did not result in any increase in mortality resulting from infection with *P. chabaudi* [356]. Similarly, *in vitro* studies utilising peritoneal exudate macrophages derived from CBA mice infected with *L. major*,

showed a synergistic increase on NO production following stimulation with IFN-γ and TNF as well as a dose-dependant decrease in leishmanicidal activity upon inhibition of NO production following treatment with the $_{L}$-arginine analogue $_{L}$-NMMA [106].

Previously, it has been shown that B6.TNF$^{-/-}$ mice suffered from a fatal visceralising form of leishmaniasis upon infection with *L. major* independently of the number of inoculating parasites [112]. Following this work, I showed that unlike susceptible mice such as BALB/c that generally failed to promote sustained IFN-γ production, TNF or TNFR1-deficient mice developed a robust IFN-γ response after infection with *L. major*. Furthermore, this response is characterized by extensive CD4^{+} T cell activation and induction of iNOS in both the footpad lesion and draining LN. However, despite systemic IFN-γ production and an associated induction of iNOS that would normally be associated with resistance, mice lacking TNF or TNFR1 still failed to limit the visceralisation of the parasite. This dissociation of IFN-γ from resistance in the absence of TNF or TNFR1 but not memTNF pointed to the transmembrane form of TNF as having a central role in linking innate leishmanicidal effector functions with the adaptive immune response. The fact that iNOS expression was intact in both TNF and TNFR1-deficient mice indicated that memTNF was acting upstream of innate phagocyte functions to promote a leishmanicidal phenotype.

This was interesting in light of the differential requirements of reactive nitrogen and oxygen species in cutaneous and visceral forms of leishmaniasis, in which iNOS played a major role in parasite control in skin lesions and draining lymph nodes, while in the spleen both iNOS and NADPH oxidase were involved [257]. My results clearly showed that TNF and TNFR1 are essential to the survival of an infection with the *L. major* isolate

MHOM/IL/81/FE/BNI. They also indicated that this susceptibility did not result from direct effects on the induction of iNOS. In fact, during the early stages of infection I could not find any major clinical differences between wildtype and TNF or TNFR1-deficient mice. Differences only became apparent approximately two weeks post infection, which correlated with the beginning of the dissemination of the parasite from cutaneous to visceral sites. Even following dissemination, while TNF-deficient mice still presented with an increasing lesion size, they showed no statistical difference in parasite burdens in the footpad, demonstrating that they still were able to exhibit partial control over parasites burden at cutaneous sites of infection.

While IFN-γ had been shown to be pivotal to protection following infection with *L. major,* a number recent findings called into question the simplicity of the IFN-γ / IL-4 paradigm of disease susceptibility. The administration of anti-IL4 mAb to susceptible BALB/c mice caused progressive uncontrolled infection of *L. major* [94] in parallel with an up-regulation of IFN-γ production. Concurrent neutralisation of this elevated IFN-γ did not change this resistance phenotype. Additionally, the generation of a number of mice congenic for known susceptibility or resistance loci (*Lmr1, Lmr2, Lmr3*), derived from either resistant C57BL/6 or susceptible BALB/c mice did not display the expected susceptible/resistant phenotype, despite expressing IL-4 or IFN-γ [151, 258, 260-262], suggesting that the IFN-γ / IL-4 cytokine profile alone was not a sufficient determinant of disease resistance. It is interesting that one of these resistance loci, *Lmr1,* spanned the region D17Mit57-D17Mit39 that encompasses the *Tnf* gene and that, depending on the donor genotype of the congene, the mice displayed either intermediate protection (C57BL/6 congene - D17Mit57-D17Mit129) or increased susceptibility (BALB/c congene - D17Mit57-D17Mit39). The similarity in susceptibility between the congenic *Lmr1* strain and the

B6.TNF$^{-/-}$ mice hinted at a potential influence on the expression on this cytokine in different susceptible and resistant strains that served to regulate some, as yet unidentified, aspect of the immune response coupling the innate and adaptive immune responses for leishmanicidal activity.

This potential regulatory activity of TNF on the inflammatory milieu is borne out through the spatial uncoupling of parasites and iNOS observed in the draining lymph node described in Chapter 5. Inflammatory monocytes characterised by the expression of CCR2, which are strong producers of TNF *in vivo* and *in vitro,* were recruited to sites of infection, co-localised with parasites in both WT and TNF-deficient mice and expressed iNOS. However, both B6.TNF- and B6.TNFR1-deficient mice additionally showed a spatial segregation of parasites and iNOS^{+} cells due to an accumulation of highly parasitised myeloid cells that did not express iNOS and formed discreet clusters within the lymph node. The development of this granular side scatter ^{high} CD11b^{+}, Ly6G^{-}, CCR2^{low}, Ly6C^{low} cells, which were a phenotypic hallmark for susceptibility in the absence of TNF, could represent an accumulation of maturing Mo-DC that down regulated CCR2 and Ly6C during maturation, either as result of an increased parasitic burden and failure to undergo apoptosis, or alternatively, through suppressive mechanisms induced by the parasite that normally would be overcome in the presence of TNF. While the exact function of these cells with regard to their role in the susceptibility to leishmaniasis is still to be determined, their ability to harbour parasites removed from leishmanicidal effector functions such as iNOS, provided a means not only for parasite escape, but also could serve in an immune modulatory capacity that facilitates parasite survival.

Mixed bone marrow chimeras utilising combinations of B6.WT, B6.TNFR1$^{-/-}$ or

B6.TNFR2$^{-/-}$ bone marrow revealed that these parasitised myeloid cells developed from both WT and TNFR1-deficient bone marrow progenitors following infection in my mixed chimera model. This argues against a direct involvement of TNF in activating leishmanicidal function, or inducing apoptosis that may explain their accumulation. Rather, their origin from both WT and TNFR1-deficient bone marrow would implicate TNF acting upstream in myeloid cell development during inflammation which would ultimately licence a cell to respond to an external stimuli to gain leishmanicidal capacity. Accordingly, in the absence of TNF/TNFR1 signalling, this would provide for a permissive environment allowing myeloid cells to be overwhelmed by parasites and perhaps succumb to a parasite / host immune evasion strategy normally not evident if TNF is present.

The clinical differences between B6.WT and B6.TNF$^{-/-}$ mice, especially during later stages of disease when visceralisation in the absence of TNF occurs, makes understanding the sequence of events that leads to the fatal outcome more difficult to reconcile. The transition from cutaneous leishmaniasis to visceral leishmaniasis in B6.TNF-deficient mice is an interesting phenomenon normally seen in susceptible strains such as BALB/c that fail to produce IFN-γ, and may result from a direct effect of TNF on promoting containment of parasites at local sites. While the mechanisims that allow for containment of *L. major* parasites at cutaneous sites were assumed to be strongly dependent on the induction of iNOS [244, 255], the failure of TNF-deficient mice to contain the parasite despite iNOS production pointed more to a TNF specific mechanism that supports containment. Indeed, pathogen containment in other disease models such as *Mycobacterium* spp. and *Leishmania donovani* relies on the formation of granulomas in a TNF-dependent manner [124, 357-359]. The dissolution of granulatomous lesions observed in the absence of

TNFR1 during *Mycobacterium avium* infection has been shown to be dependent on both IL-12 and $CD4^+$ and $CD8^+$ T cells [358], which cause a rapid dissolution of the granuloma. This facilitation of granuloma dissolution by T cells and subsequent loss of containment is remarkably similar to the events observed during the late phase of infection with *L. major*, in which a loss of parasite containment within the skin lesion and draining lymph node results in visceralisation. Notably, in the absence of TNF or TNFR1, the $CD4^+$ T cell response was greatly enhanced early in infection, both in number and activation by $CD4^+$ $V\beta4$ TCR^+ cells that contained a dominant LACK specific T cell clone compared to B6.WT. Interestingly, this observed increase in activation of $CD4^+$ T cells was not limited to this population containing putative antigenic specific T cells but spread to other non-canonical Vβ TCR chains such as Vβ5 that were not normally expanded in B6.WT mice [283]. Unfortunately, measures to address the specificity and function of antigen specific T cells using EGFP- specific IA^b tetramers (a kind gift from Dr. Mark Jenkins) and EGFP-transgenic *L. major* yielded inconclusive results (data not shown). $CD4^+$ T cells specific for eGFP were not expanded over background in all genotypes tested, raising the possibility that the expansion of $CD4^+$ $V\beta5^+$ T cells observed in the absence of TNF of TNFR1 may be representative of non-specific bystander activation as opposed to $CD4^+$ T cells raised against putative minor epitopes. Whether this increased activation of different $CD4^+$ $V\beta TCR^+$ cells in *L. major* infected TNF- or TNFR1-deficient mice is truly representative of bystander activation, or epitope spreading and its consequence to the pathology of cutaneous leishmaniasis is still to be investigated, but it is conceivable that this exacerbated T cell activation contributed to the diversion of clinical outcomes in B6.TNF and B6.TNFR1-deficient mice leading to a loss of parasite containment at cutaneous sites of infection.

The clinical manifestations in TNF- and TNFR1- deficient mice that result in visceralisation and, subsequently death could be representative of a phenomenon observed previously following *L. major* re-infection at a distal site. Under these circumstances a disequilibrium of effector and regulatory T cells developed that resulted in the migration of Tregs to sites of initial or latent infection, causing a subsequent parasite reactivation and an increased lesion size [291]. This is supported by the observations that in the absence of TNF or TNFR1, $CD4^+$, $FoxP3^{+,}$ $GITR^+$ Tregs accumulated in a linear fashion along with effector $CD4^+$ T cells. This expansion of Tregs appeared to coincide with the clinical deviation from a cutaneous to a visceral form of leishmaniasis following *L. major* infection. Thus, while a degree of parasite control may begin to be exerted in the periphery, the transition from cutaneous to visceral leishmaniasis in the absence of TNF or TNFR1 resulting from a loss of parasite containment may have similarly resulted in a comparable disequilibrium of effector and regulatory T cells that, ultimately, uncouple innate and adaptive immune responses to the parasite through altered monocyte and macrophage function. Notably, TNF-deficient mice showed an overall sustained increase in IL-10 expression that was coincident with IFN-γ expression in both the footpad and draining lymph node. The induction of IL-10, despite the ongoing IFN-γ response, may have provided a means to limit innate immune cell activation especially in macrophages, which serve as a major reservoir of *L. major* amastigotes *in vivo*. Whether this disequilibrium solely relies on expansion of Tregs in the absence of TNF, or through a cumulative cellular dysregulation is still to be fully addressed. However, results from putative Treg depletion using anti-CD25 mAb revealed that depletion of Tregs in this manner had no positive outcome on infection and that if Tregs did in fact play a role, it must occur in a CD25 independent manner.

In conclusion, the role that TNF plays in regulating inflammation and immunity in response to infection with *L. major* is complex and diverse. The fatal outcome of *L. major* infection in TNF- and TNFR1- deficient mice appears not to result from one simple aberration of immune function. Indeed, I showed that TNF affects multiple aspects of immune function, affecting migration and compartmentalisation, co-stimulation of the leishmanicidal enzyme iNOS, and modulating both T cell activation as well as the differentiation and development of myeloid cells. I have provided evidence for the dissociation of normally protective IFN-γ from innate immune function as well as the dysregulation of adaptive $CD4^+$ T cell responses that contribute to an a high degree of T cell activation across a range of different T cell specificities as well as an increase in the numebrs of Tregs through the course of infection. Finally, I have suggested that an important interaction between membrane TNF and TNFR1 exists in modulating the development of inflammatory myeloid cells that served to promote parasite clearance, and that a deficit in this pathway leads to both the development and compartmentalisation of a novel myeloid cell population which serves to segregate *L. major* amastigotes from inflammatory effector cells *in vivo*.

Further understanding the biology and the mechanisms by which memTNF and TNFR1 act upstream of direct leishmanicidial functions to promote the development of a protective immune response may also provide insight into the consequences of therapeutic targeting of TNF in autoimmune diseases and provide the basis for avoiding undesired sequela encountered from latent or re-infection with intracellular human pathogens such as *Leishmania* spp.

References

1. Fernandez, L., et al., *Tumor necrosis factor-[alpha] and endothelial cells modulate Notch signaling in the bone marrow microenvironment during inflammation.* Experimental Hematology, 2008. **36**(5): p. 545-558.e1.
2. Pearl-Yafe, M., et al., *Tumor Necrosis Factor Receptors Support Murine Hematopoietic Progenitor Function in the Early Stages of Engraftment.* Stem Cells, 2010. **28**(7): p. 1270-1280.
3. Strasser, A., P.J. Jost, and S. Nagata, *The Many Roles of FAS Receptor Signaling in the Immune System.* Immunity, 2009. **30**(2): p. 180-192.
4. Kishimoto, H., C.D. Surh, and J. Sprent, *A role for Fas in negative selection of thymocytes in vivo.* J Exp Med, 1998. **187**(9): p. 1427-38.
5. Korner, H., et al., *Distinct roles for lymphotoxin-alpha and tumor necrosis factor in organogenesis and spatial organization of lymphoid tissue.* Eur J Immunol, 1997. **27**(10): p. 2600-9.
6. Ngo, V.N., et al., *Lymphotoxin alpha /beta and Tumor Necrosis Factor Are Required for Stromal Cell Expression of Homing Chemokines in B and T Cell Areas of the Spleen.* J. Exp. Med., 1999. **189**(2): p. 403-412.
7. Aggarwal, B.B., *Signalling pathways of the TNF superfamily: a double-edged sword.* Nat Rev Immunol, 2003. **3**(9): p. 745-56.
8. Moore, R.J., et al., *Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis.* Nat Med, 1999. **5**(7): p. 828-31.
9. Arnott, C.H., et al., *Expression of both TNF-alpha receptor subtypes is essential for optimal skin tumour development.* Oncogene, 2004. **23**(10): p. 1902-10.
10. Scott, K.A., et al., *An anti-tumor necrosis factor-alpha antibody inhibits the development of experimental skin tumors.* Mol Cancer Ther, 2003. **2**(5): p. 445-51.
11. Peraldi, P., et al., *Tumor necrosis factor (TNF)-alpha inhibits insulin signaling through stimulation of the p55 TNF receptor and activation of sphingomyelinase.* J Biol Chem, 1996. **271**(22): p. 13018-22.
12. Uysal, K.T., et al., *Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function.* Nature, 1997. **389**(6651): p. 610-4.
13. Feldmann, M., *Development of anti-TNF therapy for rheumatoid arthritis.* Nat Rev Immunol, 2002. **2**(5): p. 364-71.
14. Tansey, M.G. and D.E. Szymkowski, *The TNF superfamily in 2009: new pathways, new indications, and new drugs.* Drug Discovery Today, 2009. **14**(23-24): p. 1082-1088.
15. Nedwin, G.E., et al., *Human Lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization.* Nucl. Acids Res., 1985. **13**(17): p. 6361-6373.
16. Muller, U., et al., *Tumour necrosis factor and lymphotoxin genes map close to H-2D in the mouse major histocompatibility complex.* Nature, 1987. **325**(6101): p. 265-7.
17. Milner, C.M. and R.D. Campbell, *Genetic organization of the human MHC class III region.* Front Biosci, 2001. **6**: p. D914-26.
18. Ruuls, S. and J. Sedgwick, *Unlinking tumor necrosis factor biology from the major histocompatibility complex: lessons from human genetics and animal models.* Am J Hum Genet, 1999. **65**(2): p. 294-301.

19. Ruuls, S.R., et al., *Membrane-Bound TNF Supports Secondary Lymphoid Organ Structure but Is Subservient to Secreted TNF in Driving Autoimmune Inflammation.* Immunity, 2001. **15**(4): p. 533-543.
20. Bodmer, J.-L., P. Schneider, and J.r. Tschopp, *The molecular architecture of the TNF superfamily.* Trends in Biochemical Sciences, 2002. **27**(1): p. 19-26.
21. Bazzoni, F. and B. Beutler, *The Tumor Necrosis Factor Ligand and Receptor Families.* N Engl J Med, 1996. **334**(26): p. 1717-1725.
22. Mosmann, T.R., et al., *Pillars Article: Two Types of Murine Helper T Cell Clone. I. Definition According to Profiles of Lymphokine Activities and Secreted Proteins. J. Immunol., 1986, 136: 2348-2357.* J Immunol, 2005. **175**(1): p. 5-14.
23. Walzer, T., et al., *Natural-killer cells and dendritic cells: "l'union fait la force".* Blood, 2005. **106**(7): p. 2252-2258.
24. Korner, H. and J.D. Sedgwick, *Tumour necrosis factor and lymphotoxin: molecular aspects and role in tissue-specific autoimmunity.* Immunol Cell Biol, 1996. **74**(5): p. 465-72.
25. Decker, T., M.L. Lohmann-Matthes, and G.E. Gifford, *Cell-associated tumor necrosis factor (TNF) as a killing mechanism of activated cytotoxic macrophages.* J Immunol, 1987. **138**(3): p. 957-962.
26. Zheng, Y., et al., *Evaluation of the Contribution of Different ADAMs to Tumor Necrosis Factor {alpha} (TNF{alpha}) Shedding and of the Function of the TNF{alpha} Ectodomain in Ensuring Selective Stimulated Shedding by the TNF{alpha} Convertase (TACE/ADAM17).* J. Biol. Chem., 2004. **279**(41): p. 42898-42906.
27. Gearing, A.J.H., et al., *Processing of tumour necrosis factor-[alpha] precursor by metalloproteinases.* Nature, 1994. **370**(6490): p. 555-557.
28. Moss, M.L., et al., *Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-[alpha]*. Nature, 1997. **385**(6618): p. 733-736.
29. Bell, J.H., et al., *Role of ADAM17 in the ectodomain shedding of TNF-{alpha} and its receptors by neutrophils and macrophages.* J Leukoc Biol, 2007. **82**(1): p. 173-176.
30. Black, R.A., et al., *A metalloproteinase disintegrin that releases tumour-necrosis factor-[alpha] from cells.* Nature, 1997. **385**(6618): p. 729-733.
31. Peschon, J.J., et al., *An Essential Role for Ectodomain Shedding in Mammalian Development.* Science, 1998. **282**(5392): p. 1281-1284.
32. Reddy, P., et al., *Functional Analysis of the Domain Structure of Tumor Necrosis Factor-alpha Converting Enzyme.* J. Biol. Chem., 2000. **275**(19): p. 14608-14614.
33. Garton, K.J., P.J. Gough, and E.W. Raines, *Emerging roles for ectodomain shedding in the regulation of inflammatory responses.* J Leukoc Biol, 2006. **79**(6): p. 1105-1116.
34. Solomon, K.A., et al., *Cutting Edge: A Dominant Negative Form of TNF-{alpha} Converting Enzyme Inhibits ProTNF and TNFRII Secretion.* J Immunol, 1999. **163**(8): p. 4105-4108.
35. Bemelmans, M.H., D.J. Gouma, and W.A. Buurman, *LPS-induced sTNF-receptor release in vivo in a murine model. Investigation of the role of tumor necrosis factor, IL-1, leukemia inhibiting factor, and IFN-gamma.* J Immunol, 1993. **151**(10): p. 5554-5562.
36. Tang, P., M.C. Hung, and J. Klostergaard, *Human pro-Tumor Necrosis Factor Is a Homotrimer.* Biochemistry, 1996. **35**(25): p. 8216-8225.

37. Smith, R.A. and C. Baglioni, *The active form of tumor necrosis factor is a trimer.* J. Biol. Chem., 1987. **262**(15): p. 6951-6954.
38. Wingfield, P., R.H. Pain, and S. Craig, *Tumour necrosis factor is a compact trimer.* FEBS Letters, 1987. **211**(2): p. 179-184.
39. Vandenabeele, P., et al., *Two tumour necrosis factor receptors: structure and function.* Trends in Cell Biology, 1995. **5**(10): p. 392-399.
40. Grell, M., et al., *The type 1 receptor (CD120a) is the high affinity receptor for soluble tumor necrosis factor.* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 570-575.
41. Grell, M., et al., *The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor.* Cell, 1995. **83**(5): p. 793-802.
42. Wang, C.-Y., et al., *NF-B Antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to Suppress Caspase-8 Activation.* Science, 1998. **281**(5383): p. 1680-1683.
43. Vince, J.E., et al., *TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-{kappa}b and to prevent tnf-induced apoptosis.* J Biol Chem, 2009. **284**(51): p. 35906-15.
44. Ko, Y.-G., et al., *TNF-{alpha}-Mediated Apoptosis Is Initiated in Caveolae-Like Domains.* J Immunol, 1999. **162**(12): p. 7217-7223.
45. Cottin, V., J.E.S. Doan, and D.W.H. Riches, *Restricted Localization of the TNF Receptor CD120a to Lipid Rafts: A Novel Role for the Death Domain.* J Immunol, 2002. **168**(8): p. 4095-4102.
46. Micheau, O. and J.r. Tschopp, *Induction of TNF Receptor I-Mediated Apoptosis via Two Sequential Signaling Complexes.* Cell, 2003. **114**(2): p. 181-190.
47. Baker, S.J. and E.P. Reddy, *Modulation of life and death by the TNF receptor superfamily.* Oncogene, 1998. **17**: p. 3261-3270.
48. Baud, V. and M. Karin, *Signal transduction by tumor necrosis factor and its relatives.* Trends Cell Biol, 2001. **11**(9): p. 372-377.
49. Micheau, O., et al., *NF-{kappa}B Signals Induce the Expression of c-FLIP.* Mol. Cell. Biol., 2001. **21**(16): p. 5299-5305.
50. Legler, D.F., et al., *Recruitment of TNF Receptor 1 to Lipid Rafts Is Essential for TNF alpha-Mediated NF-kappaB Activation.* Immunity, 2003. **18**(5): p. 655-664.
51. Fotin-Mleczek, M., et al., *Apoptotic crosstalk of TNF receptors: TNF-R2-induces depletion of TRAF2 and IAP proteins and accelerates TNF-R1-dependent activation of caspase-8.* J Cell Sci, 2002. **115**(13): p. 2757-2770.
52. Li, X., Y. Yang, and J.D. Ashwell, *TNF-RII and c-IAP1 mediate ubiquitination and degradation of TRAF2.* Nature, 2002. **416**(6878): p. 345-347.
53. Hehlgans, T. and K. Pfeffer, *The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games.* Immunology, 2005. **115**(1): p. 1-20.
54. Sarin, A., M. Conan-Cibotti, and P.A. Henkart, *Cytotoxic effect of TNF and lymphotoxin on T lymphoblasts.* J Immunol, 1995. **155**(8): p. 3716-3718.
55. Schutze, S., et al., *Inhibition of Receptor Internalization by Monodansylcadaverine Selectively Blocks p55 Tumor Necrosis Factor Receptor Death Domain Signaling.* J. Biol. Chem., 1999. **274**(15): p. 10203-10212.
56. Weiss, T., et al., *Enhancement of TNF receptor p60-mediated cytotoxicity by TNF receptor p80: requirement of the TNF receptor-associated factor-2 binding site.* J Immunol, 1997. **158**(5): p. 2398-404.

57. Weingartner, M., et al., *Endogenous membrane tumor necrosis factor (TNF) is a potent amplifier of TNF receptor 1-mediated apoptosis.* J Biol Chem, 2002. **277**(38): p. 34853-9.
58. Declercq, W., et al., *Cooperation of Both TNF Receptors in Inducing Apoptosis: Involvement of the TNF Receptor-Associated Factor Binding Domain of the TNF Receptor 75.* J Immunol, 1998. **161**(1): p. 390-399.
59. Chan, F.K. and M.J. Lenardo, *A crucial role for p80 TNF-R2 in amplifying p60 TNF-R1 apoptosis signals in T lymphocytes.* Eur J Immunol, 2000. **30**(2): p. 652-60.
60. Silke, J. and R. Brink, *Regulation of TNFRSF and innate immune signalling complexes by TRAFs and cIAPs.* Cell Death Differ. **17**(1): p. 35-45.
61. O'Donnell, M.A., et al., *Ubiquitination of RIP1 Regulates an NF-[kappa]B-Independent Cell-Death Switch in TNF Signaling.* Current Biology, 2007. **17**(5): p. 418-424.
62. Rauert, H., et al., *Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2).* J Biol Chem, 2009. **285**(10): p. 7394-404.
63. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 Cells: Different Patterns of Lymphokine Secretion Lead to Different Functional Properties.* Annual Review of Immunology, 1989. **7**(1): p. 145-173.
64. Szabo, S.J., et al., *A Novel Transcription Factor, T-bet, Directs Th1 Lineage Commitment.* Cell, 2000. **100**(6): p. 655-669.
65. Szabo, S.J., et al., *Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells.* Science, 2002. **295**(5553): p. 338-42.
66. Zheng, W.-p. and R.A. Flavell, *The Transcription Factor GATA-3 Is Necessary and Sufficient for Th2 Cytokine Gene Expression in CD4 T Cells.* Cell, 1997. **89**(4): p. 587-596.
67. Grogan, J.L., et al., *Early Transcription and Silencing of Cytokine Genes Underlie Polarization of T Helper Cell Subsets.* Immunity, 2001. **14**(3): p. 205-215.
68. Heinzel, F.P., et al., *Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets.* J Exp Med, 1989. **169**(1): p. 59-72.
69. Yang, X.O., et al., *T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma.* Immunity, 2008. **28**(1): p. 29-39.
70. Holmøy, T. and A.L.K. Hestvik, *Multiple sclerosis: immunopathogenesis and controversies in defining the cause.* Curr Opin Infect Dis, 2008. **21**: p. 271-278.
71. Ouyang, W., et al., *Inhibition of Th1 Development Mediated by GATA-3 through an IL-4-Independent Mechanism.* 1998. **9**(5): p. 745-755.
72. Weaver, C.T., et al., *Th17: An Effector CD4 T Cell Lineage with Regulatory T Cell Ties.* Immunity, 2006. **24**(6): p. 677-688.
73. Zakharova, M. and H.K. Ziegler, *Paradoxical Anti-Inflammatory Actions of TNF-{alpha}: Inhibition of IL-12 and IL-23 via TNF Receptor 1 in Macrophages and Dendritic Cells.* J Immunol, 2005. **175**(8): p. 5024-5033.
74. Belkaid, Y. and G. Oldenhove, *Tuning microenvironments: induction of regulatory T cells by dendritic cells.* Immunity, 2008. **29**(3): p. 362-71.
75. Chen, X., et al., *Interaction of TNF with TNF Receptor Type 2 Promotes Expansion and Function of Mouse CD4+CD25+ T Regulatory Cells.* J Immunol, 2007. **179**(1): p. 154-161.

76. Kim, E.Y. and H.-S. Teh, *TNF Type 2 Receptor (p75) Lowers the Threshold of T Cell Activation.* J Immunol, 2001. **167**(12): p. 6812-6820.
77. Kim, E.Y. and H.-S. Teh, *Critical Role of TNF Receptor Type-2 (p75) as a Costimulator for IL-2 Induction and T Cell Survival: A Functional Link to CD28.* J Immunol, 2004. **173**(7): p. 4500-4509.
78. Valencia, X., et al., *TNF downmodulates the function of human CD4+CD25hi T-regulatory cells.* Blood, 2006. **108**(1): p. 253-261.
79. Sacks, D. and N. Noben-Trauth, *The Immunology of Susceptibility and Resistance to Leishmania Major in Mice.* Nature Reviews Immunology, 2002. **2**(11): p. 845-858.
80. WHO. *Neglected Tropical Diseases*. 2010 31/07/2010 [cited 2010 03/08/2010]; Available from: http://www.who.int/neglected_diseases/en/.
81. WHO. *Cutaneous Leishmaniasis : Why are you neglecting me?* in *WHO Informal Consultative Meeting for the control of cutaneous Leishmaniasis inthe Middle East and Maghreb.* 2007. Geneva: World Health Organsiation.
82. Reithinger, R., et al., *Cutaneous leishmaniasis.* The Lancet Infectious Diseases, 2007. **7**(9): p. 581-596.
83. Bogdan, C., et al., *Fibroblasts as Host Cells in Latent Leishmaniosis.* J. Exp. Med., 2000. **191**(12): p. 2121-2130.
84. Woelbing, F., et al., *Uptake of Leishmania major by dendritic cells is mediated by Fc{gamma} receptors and facilitates acquisition of protective immunity.* J. Exp. Med., 2006. **203**(1): p. 177-188.
85. Ameen, M., *Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics.* Clinical and Experimental Dermatology, 2010.
86. Stark, D., et al., *Post-Kala-Azar Dermal Leishmaniasis Due to Leishmania infantum in a Human Immunodeficiency Virus Type 1-Infected Patient.* J. Clin. Microbiol., 2006. **44**(3): p. 1178-1180.
87. Pourahmad, M., F. Hooshmand, and M. Rahiminejad, *Cutaneous leishmaniasis associated with visceral leishmaniasis in a case of acquired immunodeficiency syndrome (AIDS).* International Journal of Dermatology, 2009. **48**(1): p. 59-61.
88. Lloyd-Smith, J.O., M. Poss, and B.T. Grenfell, *HIV-1/parasite co-infection and the emergence of new parasite strains.* Parasitology, 2008. **135**(7): p. 795-806.
89. Scott, P., et al., *Early IL-4 Production Does Not Predict Susceptibility to Leishmania major.* Experimental Parasitology, 1996. **84**(2): p. 178-187.
90. Scott, P., *IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis.* J Immunol, 1991. **147**(9): p. 3149-55.
91. Scott, P., et al., *The development of effector and memory T cells in cutaneous leishmaniasis: the implications for vaccine development.* Immunological Reviews, 2004. **201**(1): p. 318-338.
92. Morris, L., et al., *Changes in the precursor frequencies of IL-4 and IFN-gamma secreting CD4+ cells correlate with resolution of lesions in murine cutaneous leishmaniasis.* J Immunol, 1992. **149**(8): p. 2715-2721.
93. Sacks, D. and C. Anderson, *Re-examination of the immunosuppressive mechanisms mediating non-cure of Leishmania infection in mice.* Immunological Reviews, 2004. **201**(1): p. 225-238.

94. Sadick, M.D., et al., *Cure of murine leishmaniasis with anti-interleukin 4 monoclonal antibody. Evidence for a T cell-dependent, interferon gamma-independent mechanism.* J Exp Med, 1990. **171**(1): p. 115-27.
95. Chatelain, R., K. Varkila, and R. Coffman, *IL-4 induces a Th2 response in Leishmania major-infected mice.* J Immunol, 1992. **148**(4): p. 1182-1187.
96. Kopf, M., et al., *IL-4-deficient Balb/c mice resist infection with Leishmania major.* J Exp Med, 1996. **184**(3): p. 1127-36.
97. Noben-Trauth, N., W.E. Paul, and D.L. Sacks, *IL-4- and IL-4 Receptor-Deficient BALB/c Mice Reveal Differences in Susceptibility to Leishmania major Parasite Substrains.* J Immunol, 1999. **162**(10): p. 6132-6140.
98. Mohrs, M., et al., *Differences Between IL-4- and IL-4 Receptor {alpha}-Deficient Mice in Chronic Leishmaniasis Reveal a Protective Role for IL-13 Receptor Signaling.* J Immunol, 1999. **162**(12): p. 7302-7308.
99. Matthews, D.J., et al., *IL-13 Is a Susceptibility Factor for Leishmania major Infection.* J Immunol, 2000. **164**(3): p. 1458-1462.
100. Kropf, P., et al., *Factors influencing Leishmania major infection in IL-4-deficient BALB/c mice.* Parasite Immunology, 2003. **25**(8-9): p. 439-447.
101. Belkaid, Y., et al., *The Role of Interleukin (IL)-10 in the Persistence of Leishmania major in the Skin after Healing and the Therapeutic Potential of Anti-IL-10 Receptor Antibody for Sterile Cure.* J. Exp. Med., 2001. **194**(10): p. 1497-1506.
102. Kane, M.M. and D.M. Mosser, *The Role of IL-10 in Promoting Disease Progression in Leishmaniasis.* J Immunol, 2001. **166**(2): p. 1141-1147.
103. Ludewig, B., et al., *Spontaneous apoptosis of dendritic cells is efficiently inhibited by TRAP (CD40-ligand) and TNF-alpha, but strongly enhanced by interleukin-10.* Eur J Immunol, 1995. **25**(7): p. 1943-50.
104. Chang, W.L.W., et al., *Exposure of Myeloid Dendritic Cells to Exogenous or Endogenous IL-10 during Maturation Determines Their Longevity.* J Immunol, 2007. **178**(12): p. 7794-7804.
105. Raftery, M.J., et al., *Shaping Phenotype, Function, and Survival of Dendritic Cells by Cytomegalovirus-Encoded IL-10.* J Immunol, 2004. **173**(5): p. 3383-3391.
106. Liew, F.Y., Y. Li, and S. Millott, *Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of Leishmania major through the induction of nitric oxide.* J Immunol, 1990. **145**(12): p. 4306-4310.
107. Titus, R.G., B. Sherry, and A. Cerami, *Tumor necrosis factor plays a protective role in experimental murine cutaneous leishmaniasis.* J Exp Med, 1989. **170**(6): p. 2097-104.
108. Garcia, I., et al., *Transgenic mice expressing high levels of soluble TNF-R1 fusion protein are protected from lethal septic shock and cerebral malaria, and are highly sensitive to Listeria monocytogenes and Leishmania major infections.* Eur J Immunol, 1995. **25**(8): p. 2401-7.
109. Vieira, L.Q., et al., *Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with Leishmania major, but control parasite replication.* J Immunol, 1996. **157**(2): p. 827-835.
110. Nashleanas, M., S. Kanaly, and P. Scott, *Control of Leishmania major Infection in Mice Lacking TNF Receptors.* J Immunol, 1998. **160**(11): p. 5506-5513.
111. Kanaly, S.T., et al., *TNF Receptor p55 Is Required for Elimination of Inflammatory Cells Following Control of Intracellular Pathogens.* J Immunol, 1999. **163**(7): p. 3883-3889.

112. Wilhelm, P., et al., *Rapidly Fatal Leishmaniasis in Resistant C57BL/6 Mice Lacking TNF*. J Immunol, 2001. **166**(6): p. 4012-4019.
113. Allenbach, C., et al., *An essential role for transmembrane TNF in the resolution of the inflammatory lesion induced by Leishmania major infection.* European Journal of Immunology, 2008. **38**(3): p. 720-731.
114. Ritter, U., et al., *The control of Leishmania (Leishmania) major by TNF in vivo is dependent on the parasite strain.* Microbes and Infection, 2004. **6**(6): p. 559-565.
115. Smith, L.S., M. Nelson, and C.R. Dolder, *Certolizumab Pegol: A TNF-{alpha} Antagonist for the Treatment of Moderate-to-Severe Crohn's Disease.* Ann Pharmacother, 2010. **44**(2): p. 333-342.
116. Bellizzi, A., et al., *Early years of biological agents therapy in Crohn's disease and risk of the human polyomavirus JC reactivation.* Journal of Cellular Physiology. **224**(2): p. 316-326.
117. Raychaudhuri, S.P., et al., *Incidence and nature of infectious disease in patients treated with anti-TNF agents.* Autoimmunity Reviews, 2009. **9**(2): p. 67-81.
118. Keane, J., *TNF-blocking agents and tuberculosis: new drugs illuminate an old topic.* Rheumatology, 2005. **44**(6): p. 714-720.
119. De Leonardis, F., et al., *Visceral leishmaniasis and anti-TNF-alpha therapy: case report and review of the literature.* Clin Exp Rheumatol, 2009. **27**(3): p. 503-6.
120. Kritikos, K., et al., *An Atypical Presentation of Visceral Leishmaniasis Infection in a Patient With Rheumatoid Arthritis Treated With Infliximab.* JCR: Journal of Clinical Rheumatology, 2010. **16**(1): p. 38-39
121. Mueller, M.C., et al., *Relapsing Cutaneous Leishmaniasis in a Patient with Ankylosing Spondylitis Treated with Infliximab.* Am J Trop Med Hyg, 2009. **81**(1): p. 52-54.
122. Tektonidou, M.G. and F.N. Skopouli, *Visceral leishmaniasis in a patient with psoriatic arthritis treated with infliximab: reactivation of a latent infection?* Clin Rheumatol, 2008. **27**(4): p. 541-2.
123. Marino, M.W., et al., *Characterization of tumor necrosis factor-deficient mice.* Proc Natl Acad Sci U S A, 1997. **94**(15): p. 8093-8.
124. Roach, D.R., et al., *TNF Regulates Chemokine Induction Essential for Cell Recruitment, Granuloma Formation, and Clearance of Mycobacterial Infection.* J Immunol, 2002. **168**(9): p. 4620-4627.
125. Flynn, J.L., et al., *Tumor necrosis factor-[alpha] is required in the protective immune response against mycobacterium tuberculosis in mice.* Immunity, 1995. **2**(6): p. 561-572.
126. Carswell, E.A., et al., *An endotoxin-induced serum factor that causes necrosis of tumors.* Proc Natl Acad Sci U S A, 1975. **72**(9): p. 3666-70.
127. Espevik, T. and J. Nissen-Meyer, *A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes.* J Immunol Methods, 1986. **95**(1): p. 99-105.
128. Old, L.J., *Tumor necrosis factor (TNF).* Science, 1985. **230**(4726): p. 630-2.
129. Beutler, B., et al., *Identity of tumour necrosis factor and the macrophage-secreted factor cachectin.* Nature, 1985. **316**(6028): p. 552-4.
130. Kawakami, M., et al., *Lipoprotein lipase suppression in 3T3-L1 cells by an endotoxin-induced mediator from exudate cells.* Proc Natl Acad Sci U S A, 1982. **79**(3): p. 912-6.

131. Beutler, B., et al., *Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW 264.7 cells.* J Exp Med, 1985. **161**(5): p. 984-95.
132. Browning, J.L., et al., *Lymphotoxin [beta], a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface.* Cell, 1993. **72**(6): p. 847-856.
133. Nedospasov, S.A., et al., *The genes for lumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta) are tandemly arranged on chromosome 17 of the mouse.* Nucl. Acids Res., 1986. **14**(19): p. 7713-7725.
134. Kriegler, M., et al., *A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF.* Cell, 1988. **53**(1): p. 45-53.
135. Sheehan, K.C., N.H. Ruddle, and R.D. Schreiber, *Generation and characterization of hamster monoclonal antibodies that neutralize murine tumor necrosis factors.* J Immunol, 1989. **142**(11): p. 3884-93.
136. Brockhaus, M., et al., *Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies.* Proc Natl Acad Sci U S A, 1990. **87**(8): p. 3127-31.
137. Tartaglia, L.A., et al., *The two different receptors for tumor necrosis factor mediate distinct cellular responses.* Proc Natl Acad Sci U S A, 1991. **88**(20): p. 9292-6.
138. Tartaglia, L.A. and D.V. Goeddel, *Two TNF receptors.* Immunol Today, 1992. **13**(5): p. 151-3.
139. Muppidi, J.R., J. Tschopp, and R.M. Siegel, *Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction.* Immunity, 2004. **21**(4): p. 461-5.
140. Decoster, E., et al., *Generation and Biological Characterization of Membrane-bound, Uncleavable Murine Tumor Necrosis Factor.* J. Biol. Chem., 1995. **270**(31): p. 18473-18478.
141. Eck, M.J., et al., *Crystallization of trimeric recombinant human tumor necrosis factor (cachectin).* J Biol Chem, 1988. **263**(26): p. 12816-9.
142. Crowe, P.D., et al., *A lymphotoxin-beta-specific receptor.* Science, 1994. **264**(5159): p. 707-10.
143. Stauber, G.B. and B.B. Aggarwal, *Characterization and affinity cross-linking of receptors for human recombinant lymphotoxin (tumor necrosis factor-beta) on a human histiocytic lymphoma cell line, U-937.* J Biol Chem, 1989. **264**(6): p. 3573-6.
144. Aversa, G., J. Punnonen, and J.E. de Vries, *The 26-kD transmembrane form of tumor necrosis factor alpha on activated CD4+ T cell clones provides a costimulatory signal for human B cell activation.* J Exp Med, 1993. **177**(6): p. 1575-85.
145. Mueller, C., et al., *Noncleavable Transmembrane Mouse Tumor Necrosis Factor-alpha (TNFalpha) Mediates Effects Distinct from Those of Wild-type TNFalpha in Vitro and in Vivo.* J. Biol. Chem., 1999. **274**(53): p. 38112-38118.
146. McDermott, M.F., et al., *Germline Mutations in the Extracellular Domains of the 55 kDa TNF Receptor, TNFR1, Define a Family of Dominantly Inherited Autoinflammatory Syndromes.* Cell, 1999. **97**(1): p. 133-144.

147. Pocsik, E., E. Duda, and D. Wallach, *Phosphorylation of the 26 kDa TNF precursor in monocytic cells and in transfected HeLa cells.* J Inflamm, 1995. **45**(3): p. 152-60.
148. Watts, A.D., et al., *A casein kinase I motif present in the cytoplasmic domain of members of the tumour necrosis factor ligand family is implicated in 'reverse signalling'.* EMBO J, 1999. **18**(8): p. 2119-26.
149. Higuchi, M., et al., *Membrane Tumor Necrosis Factor-[alpha] (TNF-[alpha]) Expressed on HTLV-I-Infected T Cells Mediates a Costimulatory Signal for B Cell Activation--Characterization of Membrane TNF-[alpha].* Clin Immunol Immunopathol, 1997. **82**(2): p. 133-140.
150. Lipoldova, M., et al., *Suscepibility to Leishmania major infection in mice: multiple loci and hetrogeneity of imunopathological phenotypes.* Genes and Immunity, 2000(1): p. 200-206.
151. Sakthianandeswaren, A., S.J. Foote, and E. Handman, *The role of host genetics in leishmaniasis.* Trends Parasitol, 2009. **25**(8): p. 383-91.
152. Williams, D.M., F.C. Grumet, and J.S. Remington, *Genetic control of murine resistance to Toxoplasma gondii.* Infect Immun, 1978. **19**(2): p. 416-20.
153. Stevenson, M., S. Lemieux, and E. Skamene, *Genetic control of resistance to murine malaria.* J Cell Biochem, 1984. **24**(1): p. 91-102.
154. de Souza, J.B. and E.M. Riley, *Cerebral malaria: the contribution of studies in animal models to our understanding of immunopathogenesis.* Microbes Infect, 2002. **4**(3): p. 291-300.
155. Lou, J., R. Lucas, and G.E. Grau, *Pathogenesis of cerebral malaria: recent experimental data and possible applications for humans.* Clin Microbiol Rev, 2001. **14**(4): p. 810-20.
156. Clark, K., et al., *Lymphotoxin alpha and tumour necrosis factor are not required for control of parasite growth, but differentially regulate cytokine production during Plasmodium chabaudi chabaudi AS infection.* Parasite Immunology, 2007. **29**(3): p. 153-158.
157. Jacobs, P., D. Radzioch, and M.M. Stevenson, *A Th1-associated increase in tumor necrosis factor alpha expression in the spleen correlates with resistance to blood-stage malaria in mice.* Infect Immun, 1996. **64**(2): p. 535-41.
158. Li, C., et al., *Pathology of Plasmodium chabaudi chabaudi infection and mortality in interleukin-10-deficient mice are ameliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor beta antibodies.* Infect Immun, 2003. **71**(9): p. 4850-6.
159. Engwerda, C.R., et al., *A Role for Tumor Necrosis Factor-{alpha} in Remodeling the Splenic Marginal Zone during Leishmania donovani Infection.* Am J Pathol, 2002. **161**(2): p. 429-437.
160. Parekh, S.B., et al., *Brain metabolic markers reflect susceptibility status in cytokine gene knockout mice with murine cerebral malaria.* Int J Parasitol, 2006. **36**(13): p. 1409-18.
161. Hernandez-Valladares, M., et al., *Pathology of Tnf-deficient mice infected with Plasmodium chabaudi adami 408XZ.* Exp Parasitol, 2006. **114**(4): p. 271-8.
162. Grau, G.E., et al., *Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria.* Science, 1987. **237**(4819): p. 1210-2.

163. Postma, N.S., et al., *Treatment with recombinant human tumour necrosis factor-alpha reduces parasitaemia and prevents Plasmodium berghei K173-induced experimental cerebral malaria in mice.* Parasitology, 1999. **118 (Pt 1)**: p. 7-15.
164. Clark, I.A., et al., *Inhibition of murine malaria (Plasmodium chabaudi) in vivo by recombinant interferon-gamma or tumor necrosis factor, and its enhancement by butylated hydroxyanisole.* J Immunol, 1987. **139**(10): p. 3493-6.
165. Taverne, J., et al., *Anaemia and resistance to malaria in transgenic mice expressing human tumour necrosis factor.* Immunology, 1994. **82**(3): p. 397-403.
166. Rudin, W., et al., *Resistance to cerebral malaria in tumor necrosis factor-alpha/beta-deficient mice is associated with a reduction of intercellular adhesion molecule-1 up-regulation and T helper type 1 response.* Am J Pathol, 1997. **150**(1): p. 257-66.
167. Li, C. and J. Langhorne, *Tumor necrosis factor alpha p55 receptor is important for development of memory responses to blood-stage malaria infection.* Infect Immun, 2000. **68**(10): p. 5724-30.
168. Lucas, R., et al., *Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malaria.* Eur J Immunol, 1997. **27**(7): p. 1719-25.
169. Lucas, R., et al., *Respective role of TNF receptors in the development of experimental cerebral malaria.* J Neuroimmunol, 1997. **72**(2): p. 143-8.
170. Piguet, P.F., C.D. Kan, and C. Vesin, *Role of the tumor necrosis factor receptor 2 (TNFR2) in cerebral malaria in mice.* Lab Invest, 2002. **82**(9): p. 1155-66.
171. Sam, H., Z. Su, and M.M. Stevenson, *Deficiency in tumor necrosis factor alpha activity does not impair early protective Th1 responses against blood-stage malaria.* Infect Immun, 1999. **67**(5): p. 2660-4.
172. Orengo, J.M., et al., *Uric acid is a mediator of the Plasmodium falciparum-induced inflammatory response.* PLoS One, 2009. **4**(4): p. e5194.
173. Parroche, P., et al., *Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9.* Proc Natl Acad Sci U S A, 2007. **104**(6): p. 1919-24.
174. Pichyangkul, S., P. Saengkrai, and H.K. Webster, *Plasmodium falciparum pigment induces monocytes to release high levels of tumor necrosis factor-alpha and interleukin-1 beta.* Am J Trop Med Hyg, 1994. **51**(4): p. 430-5.
175. Schofield, L. and F. Hackett, *Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites.* J Exp Med, 1993. **177**(1): p. 145-53.
176. Grau, G.E., et al., *Monoclonal antibody against interferon gamma can prevent experimental cerebral malaria and its associated overproduction of tumor necrosis factor.* Proc Natl Acad Sci U S A, 1989. **86**(14): p. 5572-4.
177. Scuderi, P., et al., *Raised serum levels of tumour necrosis factor in parasitic infections.* Lancet, 1986. **2**(8520): p. 1364-5.
178. Brown, H., et al., *Cytokine expression in the brain in human cerebral malaria.* J Infect Dis, 1999. **180**(5): p. 1742-6.
179. Grau, G.E., et al., *Tumor necrosis factor and disease severity in children with falciparum malaria.* N Engl J Med, 1989. **320**(24): p. 1586-91.
180. Kwiatkowski, D., et al., *TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated Plasmodium falciparum malaria.* Lancet, 1990. **336**(8725): p. 1201-4.

181. Fried, M., et al., *Malaria elicits type 1 cytokines in the human placenta: IFN-gamma and TNF-alpha associated with pregnancy outcomes.* J Immunol, 1998. **160**(5): p. 2523-30.
182. Knight, J.C., et al., *A polymorphism that affects OCT-1 binding to the TNF promoter region is associated with severe malaria.* Nat Genet, 1999. **22**(2): p. 145-50.
183. McGuire, W., et al., *Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria.* Nature, 1994. **371**(6497): p. 508-10.
184. van Hensbroek, M.B., et al., *The effect of a monoclonal antibody to tumor necrosis factor on survival from childhood cerebral malaria.* J Infect Dis, 1996. **174**(5): p. 1091-7.
185. Jennings, V.M., et al., *Cytokine profile suggesting that murine cerebral malaria is an encephalitis.* Infect Immun, 1997. **65**(11): p. 4883-7.
186. Medana, I.M., N.H. Hunt, and G. Chaudhri, *Tumor necrosis factor-alpha expression in the brain during fatal murine cerebral malaria: evidence for production by microglia and astrocytes.* Am J Pathol, 1997. **150**(4): p. 1473-86.
187. van der Heyde, H.C., et al., *A unified hypothesis for the genesis of cerebral malaria: sequestration, inflammation and hemostasis leading to microcirculatory dysfunction.* Trends Parasitol, 2006. **22**(11): p. 503-8.
188. Descamps, L., R. Cecchelli, and G. Torpier, *Effects of tumor necrosis factor on receptor-mediated endocytosis and barrier functions of bovine brain capillary endothelial cell monolayers.* J Neuroimmunol, 1997. **74**(1-2): p. 173-84.
189. Poovassery, J.S., et al., *Malaria-induced murine pregnancy failure: distinct roles for IFN-gamma and TNF.* J Immunol, 2009. **183**(8): p. 5342-9.
190. Mackintosh, C.L., J.G. Beeson, and K. Marsh, *Clinical features and pathogenesis of severe malaria.* Trends Parasitol, 2004. **20**(12): p. 597-603.
191. Lamb, T.J., et al., *Insights into the immunopathogenesis of malaria using mouse models.* Expert Rev Mol Med, 2006. **8**(6): p. 1-22.
192. Cross, C.E. and J. Langhorne, *Plasmodium chabaudi chabaudi (AS): inflammatory cytokines and pathology in an erythrocytic-stage infection in mice.* Exp Parasitol, 1998. **90**(3): p. 220-9.
193. Seixas, E. and D. Ostler, *Plasmodium chabaudi chabaudi (AS): differential cellular responses to infection in resistant and susceptible mice.* Exp Parasitol, 2005. **110**(4): p. 394-405.
194. Seixas, E., et al., *An experimental model for fatal malaria due to TNF-alpha-dependent hepatic damage.* Parasitology, 2008. **135**(6): p. 683-90.
195. Stevenson, M.M. and E. Ghadirian, *Human recombinant tumor necrosis factor alpha protects susceptible A/J mice against lethal Plasmodium chabaudi AS infection.* Infect Immun, 1989. **57**(12): p. 3936-9.
196. Kern, P., et al., *Soluble tumor necrosis factor receptors correlate with parasitemia and disease severity in human malaria.* J Infect Dis, 1992. **166**(4): p. 930-4.
197. McGuire, W., et al., *Levels of tumour necrosis factor and soluble TNF receptors during malaria fever episodes in the community.* Trans R Soc Trop Med Hyg, 1998. **92**(1): p. 50-3.
198. Long, G.H., et al., *Blockade of TNF receptor 1 reduces disease severity but increases parasite transmission during Plasmodium chabaudi chabaudi infection.* Int J Parasitol, 2008. **38**(8-9): p. 1073-81.

199. Stoelcker, B., et al., *Requirement for tumor necrosis factor receptor 2 expression on vascular cells to induce experimental cerebral malaria.* Infect Immun, 2002. **70**(10): p. 5857-9.
200. Togbe, D.e., et al., *Both Functional LTŒ≤ Receptor and TNF Receptor 2 Are Required for the Development of Experimental Cerebral Malaria.* PLoS One, 2008. **3**(7): p. e2608.
201. Minigo, G., et al., *Parasite-dependent expansion of TNF receptor II-positive regulatory T cells with enhanced suppressive activity in adults with severe malaria.* PLoS Pathog, 2009. **5**(4): p. e1000402.
202. Walther, M., et al., *Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe Plasmodium falciparum malaria.* PLoS Pathog, 2009. **5**(4): p. e1000364.
203. Montoya, J.G. and O. Liesenfeld, *Toxoplasmosis.* Lancet, 2004. **363**(9425): p. 1965-76.
204. Ferguson, D.J. and W.M. Hutchison, *The host-parasite relationship of Toxoplasma gondii in the brains of chronically infected mice.* Virchows Arch A Pathol Anat Histopathol, 1987. **411**(1): p. 39-43.
205. Luft, B.J., et al., *Toxoplasmic encephalitis in patients with acquired immune deficiency syndrome.* JAMA, 1984. **252**(7): p. 913-7.
206. Luft, B.J. and J.S. Remington, *Toxoplasmic encephalitis in AIDS.* Clin Infect Dis, 1992. **15**(2): p. 211-22.
207. Suzuki, Y., et al., *Induction of tumor necrosis factor-alpha and inducible nitric oxide synthase fails to prevent toxoplasmic encephalitis in the absence of interferon-gamma in genetically resistant BALB/c mice.* Microbes Infect, 2000. **2**(5): p. 455-62.
208. Debierre-Grockiego, F., et al., *Fatty acids isolated from Toxoplasma gondii reduce glycosylphosphatidylinositol-induced tumor necrosis factor alpha production through inhibition of the NF-kappaB signaling pathway.* Infect Immun, 2007. **75**(6): p. 2886-93.
209. Bennouna, S., et al., *Cross-talk in the innate immune system: neutrophils instruct recruitment and activation of dendritic cells during microbial infection.* J Immunol, 2003. **171**(11): p. 6052-8.
210. Denkers, E.Y., et al., *Neutrophils, dendritic cells and Toxoplasma.* Int J Parasitol, 2004. **34**(3): p. 411-21.
211. Brown, C.R., et al., *Definitive identification of a gene that confers resistance against Toxoplasma cyst burden and encephalitis.* Immunology, 1995. **85**(3): p. 419-28.
212. Johnson, L.L., *A protective role for endogenous tumor necrosis factor in Toxoplasma gondii infection.* Infect Immun, 1992. **60**(5): p. 1979-83.
213. Gazzinelli, R.T., et al., *Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-alpha and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation.* J Immunol, 1993. **151**(7): p. 3672-81.
214. Deckert-Schluter, M., et al., *Crucial role of TNF receptor type 1 (p55), but not of TNF receptor type 2 (p75), in murine toxoplasmosis.* J Immunol, 1998. **160**(7): p. 3427-36.

215. Schluter, D., et al., *Both lymphotoxin-alpha and TNF are crucial for control of Toxoplasma gondii in the central nervous system.* J Immunol, 2003. **170**(12): p. 6172-82.
216. Deckert-Schluter, M., et al., *Toxoplasma encephalitis in congenic B10 and BALB mice: impact of genetic factors on the immune response.* Infect Immun, 1994. **62**(1): p. 221-8.
217. Freund, Y.R., et al., *Polymorphisms in the tumor necrosis factor alpha (TNF-alpha) gene correlate with murine resistance to development of toxoplasmic encephalitis and with levels of TNF-alpha mRNA in infected brain tissue.* J Exp Med, 1992. **175**(3): p. 683-8.
218. Subauste, C.S. and M. Wessendarp, *CD40 restrains in vivo growth of Toxoplasma gondii independently of gamma interferon.* Infect Immun, 2006. **74**(3): p. 1573-9.
219. Yap, G.S., et al., *Decreased resistance of TNF receptor p55- and p75-deficient mice to chronic toxoplasmosis despite normal activation of inducible nitric oxide synthase in vivo.* J Immunol, 1998. **160**(3): p. 1340-5.
220. Lassoued, S., et al., *Toxoplasmic chorioretinitis and antitumor necrosis factor treatment in rheumatoid arthritis.* Semin Arthritis Rheum, 2007. **36**(4): p. 262-3.
221. Barrett, M.P., et al., *The trypanosomiases.* Lancet, 2003. **362**(9394): p. 1469-80.
222. Sternberg, J.M., *Human African trypanosomiasis: clinical presentation and immune response.* Parasite Immunol, 2004. **26**(11-12): p. 469-76.
223. Teixeira, M.M., R.T. Gazzinelli, and J.S. Silva, *Chemokines, inflammation and Trypanosoma cruzi infection.* Trends Parasitol, 2002. **18**(6): p. 262-5.
224. Denkers, E.Y. and B.A. Butcher, *Sabotage and exploitation in macrophages parasitized by intracellular protozoans.* Trends Parasitol, 2005. **21**(1): p. 35-41.
225. Magez, S., et al., *The glycosyl-inositol-phosphate and dimyristoylglycerol moieties of the glycosylphosphatidylinositol anchor of the trypanosome variant-specific surface glycoprotein are distinct macrophage-activating factors.* J Immunol, 1998. **160**(4): p. 1949-56.
226. Camargo, M.M., et al., *Glycosylphosphatidylinositol-anchored mucin-like glycoproteins isolated from Trypanosoma cruzi trypomastigotes initiate the synthesis of proinflammatory cytokines by macrophages.* J Immunol, 1997. **158**(12): p. 5890-901.
227. Munoz-Fernandez, M.A., M.A. Fernandez, and M. Fresno, *Synergism between tumor necrosis factor-alpha and interferon-gamma on macrophage activation for the killing of intracellular Trypanosoma cruzi through a nitric oxide-dependent mechanism.* Eur J Immunol, 1992. **22**(2): p. 301-7.
228. Daulouede, S., et al., *Human macrophage tumor necrosis factor (TNF)-alpha production induced by Trypanosoma brucei gambiense and the role of TNF-alpha in parasite control.* J Infect Dis, 2001. **183**(6): p. 988-91.
229. Silva, J.S., et al., *Tumor necrosis factor alpha mediates resistance to Trypanosoma cruzi infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages.* Infect Immun, 1995. **63**(12): p. 4862-7.
230. Magez, S., et al., *Specific uptake of tumor necrosis factor-alpha is involved in growth control of Trypanosoma brucei.* J Cell Biol, 1997. **137**(3): p. 715-27.
231. Lucas, R., et al., *Mapping the lectin-like activity of tumor necrosis factor.* Science, 1994. **263**(5148): p. 814-7.

232. Black, C.M., et al., *Effect of recombinant tumour necrosis factor on acute infection in mice with Toxoplasma gondii or Trypanosoma cruzi.* Immunology, 1989. **68**(4): p. 570-4.
233. Truyens, C., et al., *The cachexia associated with Trypanosoma cruzi acute infection in mice is attenuated by anti-TNF-alpha, but not by anti-IL-6 or anti-IFN-gamma antibodies.* Parasite Immunol, 1995. **17**(11): p. 561-8.
234. Truyens, C., et al., *The endogenous balance of soluble tumor necrosis factor receptors and tumor necrosis factor modulates cachexia and mortality in mice acutely infected with Trypanosoma cruzi.* Infect Immun, 1999. **67**(11): p. 5579-86.
235. Magez, S., et al., *P75 tumor necrosis factor-receptor shedding occurs as a protective host response during African trypanosomiasis.* J Infect Dis, 2004. **189**(3): p. 527-39.
236. Castanos-Velez, E., et al., *Trypanosoma cruzi infection in tumor necrosis factor receptor p55-deficient mice.* Infect Immun, 1998. **66**(6): p. 2960-8.
237. Magez, S., et al., *Tumor necrosis factor alpha is a key mediator in the regulation of experimental Trypanosoma brucei infections.* Infect Immun, 1999. **67**(6): p. 3128-32.
238. Magez, S., et al., *Control of experimental Trypanosoma brucei infections occurs independently of lymphotoxin-alpha induction.* Infect Immun, 2002. **70**(3): p. 1342-51.
239. Iraqi, F., et al., *Susceptibility of tumour necrosis factor-alpha genetically deficient mice to Trypanosoma congolense infection.* Parasite Immunol, 2001. **23**(8): p. 445-51.
240. Sacks, D.L. and P.V. Perkins, *Identification of an infective state of Leishmania promastigotes.* Science, 1984. **v223**: p. p1417(3).
241. Sacks, D.L. and P.V. Perkins, *Development of Infective Stage Leishmania Promastigotes within Phlebotomine Sand Flies.* Am J Trop Med Hyg, 1985. **34**(3): p. 456-459.
242. Bogdan, C. and C. Nathan, *Modulation of Macrophage Function by Transforming Growth Factor Beta;, Interleukin-4, and Interleukin-10a.* Annals of the New York Academy of Sciences, 1993. **685**(Immunomodulating Drugs): p. 713-739.
243. Murray, H.W., *Interaction of Leishmania with a macrophage cell line. Correlation between intracellular killing and the generation of oxygen intermediates.* J Exp Med, 1981. **153**(6): p. 1690-5.
244. Stenger, S., et al., *Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase.* J Exp Med, 1996. **183**(4): p. 1501-14.
245. Liew, F.Y., et al., *TNF-alpha reverses the disease-exacerbating effect of subcutaneous immunization against murine cutaneous leishmaniasis.* Immunology, 1991. **74**(2): p. 304-9.
246. de Kossodo, S., et al., *Tumor necrosis factor alpha (TNF-alpha) and TNF-beta and their receptors in experimental cutaneous leishmaniasis.* Infect. Immun., 1994. **62**(4): p. 1414-1420.
247. Theodos, C.M., et al., *Role of tumor necrosis factor in macrophage leishmanicidal activity in vitro and resistance to cutaneous leishmaniasis in vivo.* Infect Immun, 1991. **59**(8): p. 2839-42.
248. Franklin, G., J. Greenspan, and S. Chen, *Anti-tumor necrosis factor-alpha therapy provokes latent leishmaniasis in a patient with rheumatoid arthritis.* Ann Clin Lab Sci, 2009. **39**(2): p. 192-5.

249. Wilhelm, P., et al., *Membrane lymphotoxin contributes to anti-leishmanial immunity by controlling structural integrity of lymphoid organs.* Eur J Immunol, 2002. **32**(7): p. 1993-2003.
250. Tumang, M.C., et al., *Role and effect of TNF-alpha in experimental visceral leishmaniasis.* J Immunol, 1994. **153**(2): p. 768-775.
251. Schonian, G., et al., *Leishmaniases in the Mediterranean in the era of molecular epidemiology.* Trends in Parasitology, 2008. **24**(3): p. 135-142.
252. Bogdan, C., *Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example.* Cell Microbiol, 2008. **10**(6): p. 1221-34.
253. Liese, J., U. Schleicher, and C. Bogdan, *The innate immune response against Leishmania parasites.* Immunobiology, 2008. **213**(3-4): p. 377-387.
254. Scharton-Kersten, T., et al., *IL-12 is required for natural killer cell activation and subsequent T helper 1 cell development in experimental leishmaniasis.* J Immunol, 1995. **154**(10): p. 5320-5330.
255. Diefenbach, A., et al., *Type 1 Interferon (IFN[alpha]/[beta]) and Type 2 Nitric Oxide Synthase Regulate the Innate Immune Response to a Protozoan Parasite.* Immunity, 1998. **8**(1): p. 77-87.
256. Murray, H.W. and C.F. Nathan, *Macrophage Microbicidal Mechanisms In Vivo: Reactive Nitrogen versus Oxygen Intermediates in the Killing of Intracellular Visceral Leishmania donovani.* J. Exp. Med., 1999. **189**(4): p. 741-746.
257. Blos, M., et al., *Organ-specific and stage-dependent control of Leishmania major infection by inducible nitric oxide synthase and phagocyte NADPH oxidase.* Eur J Immunol, 2003. **33**(5): p. 1224-1234.
258. Baldwin, T., et al., *Wound healing response is a major contributor to the severity of cutaneous leishmaniasis in the ear model of infection.* Parasite Immunol, 2007. **29**(10): p. 501-13.
259. Sakthianandeswaren, A., et al., *Fine mapping of Leishmania major susceptibility locus lmr2: evidence for a role for Fli1 in disease and wound healing.* Infect Immun, 2010.
260. Sakthianandeswaren, A., et al., *The wound repair response controls outcome to cutaneous leishmaniasis.* Proc Natl Acad Sci U S A, 2005. **102**(43): p. 15551-6.
261. Elso, C., et al., *Dissociation of disease susceptibility, inflammation and cytokine profile in lmr1/2 congenic mice infected with Leishmania major.* Genes Immun, 2004. **5**(3): p. 188-96.
262. Elso, C.M., et al., *Leishmaniasis host response loci (lmr1-3) modify disease severity through a Th1/Th2-independent pathway.* Genes Immun, 2004. **5**(2): p. 93-100.
263. Belosevic, M., et al., *Administration of monoclonal anti-IFN-gamma antibodies in vivo abrogates natural resistance of C3H/HeN mice to infection with Leishmania major.* J Immunol, 1989. **143**(1): p. 266-274.
264. Green, S.J., et al., *Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha.* J Immunol, 1990. **145**(12): p. 4290-4297.
265. Peschon, J.J., et al., *TNF Receptor-Deficient Mice Reveal Divergent Roles for p55 and p75 in Several Models of Inflammation.* J Immunol, 1998. **160**(2): p. 943-952.
266. Solbach, W., et al., *Suppressive effect of cyclosporin A on the development of Leishmania tropica-induced lesions in genetically susceptible BALB/c mice.* J Immunol, 1986. **137**(2): p. 702-707.

267. Schleicher, U. and C. Bogdan, *Generation, culture and flow-cytometric characterization of primary mouse macrophages.* Methods Mol Biol, 2009. **531**: p. 203-24.
268. Meissner, A., et al., *CC chemokine ligand 20 partially controls adhesion of naive B cells to activated endothelial cells under shear stress.* Blood, 2003. **102**(8): p. 2724-2727.
269. Shoemaker, J., M. Saraiva, and A. O'Garra, *GATA-3 Directly Remodels the IL-10 Locus Independently of IL-4 in CD4+ T Cells.* J Immunol, 2006. **176**(6): p. 3470-3479.
270. Korner, H., et al., *The role of TNF in parasitic diseases: Still more questions than answers.* Int J Parasitol, 2010. **40**(8): p. 879-888.
271. Heinzel, F.P., et al., *Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis.* Proc Natl Acad Sci U S A, 1991. **88**(16): p. 7011-5.
272. Wilhelm, P., et al., *TNF but not Fas ligand provides protective anti-L. major immunity in C57BL/6 mice.* Microbes and Infection, 2005. **7**(15): p. 1461-1468.
273. Schroder, K., M.J. Sweet, and D.A. Hume, *Signal integration between IFN gamma and TLR signalling pathways in macrophages.* Immunobiology, 2006. **211**(6-8): p. 511-524.
274. Held, T., et al., *Gamma interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1.* Infect Immun, 1999(0019-9567 (Print)).
275. Saito, S. and M. Nakano, *Nitric oxide production by peritoneal macrophages of Mycobacterium bovis BCG-infected or non-infected mice: regulatory role of T lymphocytes and cytokines.* J Leukoc Biol, 1996. **59**(6): p. 908-915.
276. Swihart, K., et al., *Mice from a genetically resistant background lacking the interferon gamma receptor are susceptible to infection with Leishmania major but mount a polarized T helper cell 1-type CD4+ T cell response.* J Exp Med, 1995. **181**(3): p. 961-71.
277. Wang, Z.E., et al., *CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with Leishmania major.* J Exp Med, 1994. **179**(4): p. 1367-71.
278. Ohmori, Y., R. Schreiber, and T.A. Hamilton, *Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB.* J. Biol. Chem, 1997. **272**(0021-9258 (Print)): p. 14899-14907.
279. Saura, M., et al., *Interaction of interferon regulatory factor-1 and nuclear factor [kappa]B during activation of inducible nitric oxide synthase transcription.* Journal of Molecular Biology, 1999. **289**(3): p. 459-471.
280. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-189.
281. Stenger, S., et al., *Tissue expression of inducible nitric oxide synthase is closely associated with resistance to Leishmania major.* J Exp Med, 1994. **180**(3): p. 783-93.

282. Sundquist, M. and M.J. Wick, *TNF-{alpha}-Dependent and -Independent Maturation of Dendritic Cells and Recruited CD11cintCD11b+ Cells during Oral Salmonella Infection.* J Immunol, 2005. **175**(5): p. 3287-3298.
283. Reiner, S.L., et al., *TH1 and TH2 Cell Antigen Receptors in Experimental Leishmaniasis.* Science, 1993. **259**(5100): p. 1457-1460.
284. Kamala, T. and N.K. Nanda, *Protective Response to Leishmania major in BALB/c Mice Requires Antigen Processing in the Absence of DM.* J Immunol, 2009. **182**(8): p. 4882-4890.
285. Julia, V. and N. Glaichenhaus, *CD4(+) T cells which react to the Leishmania major LACK antigen rapidly secrete interleukin-4 and are detrimental to the host in resistant B10.D2 mice.* Infect Immun, 1999. **67**(7): p. 3641-4.
286. Launois, P., et al., *In susceptible mice, Leishmania major induce very rapid interleukin-4 production by CD4+ T cells which are NK1.1.* Eur J Immunol, 1995. **25**(12): p. 3298-307.
287. Kelly, B.L. and R.M. Locksley, *The Leishmania major LACK Antigen with an Immunodominant Epitope at Amino Acids 156 to 173 Is Not Required for Early Th2 Development in BALB/c Mice.* Infect. Immun., 2004. **72**(12): p. 6924-6931.
288. Launois, P., et al., *Different epitopes of the LACK protein are recognized by V beta 4 V alpha 8 CD4+ T cells in H-2b and H-2d mice susceptible to Leishmania major.* Microbes Infect, 2007. **9**(11): p. 1260-6.
289. Evangelidou, M., et al., *TNFRI is a positive T-cell costimulatory molecule important for the timing of cytokine responses.* Immunol Cell Biol, 2010. **88**(5): p. 586-95.
290. Misslitz, A., et al., *Targeted integration into a rRNA locus results in uniform and high level expression of transgenes in Leishmania amastigotes.* Molecular and Biochemical Parasitology, 2000. **107**(2): p. 251-261.
291. Mendez, S., et al., *Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity.* J Exp Med, 2004. **200**(2): p. 201-10.
292. Lyons, A.B. and C.R. Parish, *Determination of lymphocyte division by flow cytometry.* Journal of Immunological Methods, 1994. **171**(1): p. 131-137.
293. Sakon, S., et al., *NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death.* EMBO J, 2003. **22**(15): p. 3898-909.
294. Kobayashi, T., et al., *TRAF6 Is a Critical Factor for Dendritic Cell Maturation and Development.* Immunity, 2003. **19**(3): p. 353-363.
295. Ritter, U., et al., *Analysis of the maturation process of dendritic cells deficient for TNF and lymphotoxin-{alpha} reveals an essential role for TNF.* J Leukoc Biol, 2003. **74**(2): p. 216-222.
296. Chomarat, P., et al., *TNF Skews Monocyte Differentiation from Macrophages to Dendritic Cells.* J Immunol, 2003. **171**(5): p. 2262-2269.
297. Thomas, R.M., et al., *Ikaros silences T-bet expression and interferon-gamma production during T helper 2 differentiation.* J Biol Chem, 2010. **285**(4): p. 2545-53.
298. Fathman, C.G. and N.B. Lineberry, *Molecular mechanisms of CD4+ T-cell anergy.* Nat Rev Immunol, 2007. **7**(8): p. 599-609.

299. Legge, K.L. and T.J. Braciale, *Lymph Node Dendritic Cells Control CD8+ T Cell Responses through Regulated FasL Expression.* Immunity, 2005. **23**(6): p. 649-659.
300. Launois, P., et al., *IL-4 Rapidly Produced by V[beta]4 V[alpha]8 CD4+ T Cells Instructs Th2 Development and Susceptibility to Leishmania major in BALB/c Mice.* Immunity, 1997. **6**(5): p. 541-549.
301. Holaday, B.J., et al., *Reconstitution of Leishmania immunity in severe combined immunodeficient mice using Th1- and Th2-like cell lines.* J Immunol, 1991. **147**(5): p. 1653-1658.
302. Sypek, J.P. and D.J. Wyler, *T-cell hybridomas reveal two distinct mechanisms of antileishmanial defense.* Infect Immun, 1990. **58**(5): p. 1146-52.
303. Belkaid, Y., et al., *CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity.* Nature, 2002. **420**(6915): p. 502-507.
304. Anderson, C.F., et al., *CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis.* J Exp Med, 2007. **204**(2): p. 285-97.
305. Viana da Costa, A., et al., *IL-10 leads to a higher parasite persistence in a resistant mouse model of Leishmania major infection.* Parasitology International, 2002. **51**(4): p. 367-379.
306. Vila-del Sol, V., M.D. Diaz-Munoz, and M. Fresno, *Requirement of tumor necrosis factor {alpha} and nuclear factor-{kappa}B in the induction by IFN-{gamma} of inducible nitric oxide synthase in macrophages.* J Leukoc Biol, 2007. **81**(1): p. 272-283.
307. Bekker, L.-G., et al., *TNF-alpha Controls Intracellular Mycobacterial Growth by Both Inducible Nitric Oxide Synthase-Dependent and Inducible Nitric Oxide Synthase-Independent Pathways.* J Immunol, 2001. **166**(11): p. 6728-6734.
308. Calder, C.J., L.B. Nicholson, and A.D. Dick, *A Selective Role for the TNF p55 Receptor in Autocrine Signaling following IFN-{gamma} Stimulation in Experimental Autoimmune Uveoretinitis.* J Immunol, 2005. **175**(10): p. 6286-6293.
309. Fonseca, S.G., et al., *TNF-alpha mediates the induction of nitric oxide synthase in macrophages but not in neutrophils in experimental cutaneous leishmaniasis.* European Journal of Immunology, 2003. **33**(8): p. 2297-2306.
310. Engel, D., et al., *Tumor Necrosis Factor Alpha- and Inducible Nitric Oxide Synthase-Producing Dendritic Cells Are Rapidly Recruited to the Bladder in Urinary Tract Infection but Are Dispensable for Bacterial Clearance.* Infect. Immun., 2006. **74**(11): p. 6100-6107.
311. Leon, B., M. Lopez-Bravo, and C. Ardavin, *Monocyte-Derived Dendritic Cells Formed at the Infection Site Control the Induction of Protective T Helper 1 Responses against Leishmania.* Immunity, 2007. **26**(4): p. 519-531.
312. De Trez, C., et al., *iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic Leishmania major infection phase of C57BL/6 resistant mice.* PLoS Pathog, 2009. **5**(6): p. 1553-7374 (Electronic).
313. Geissmann, F., S. Jung, and D.R. Littman, *Blood Monocytes Consist of Two Principal Subsets with Distinct Migratory Properties.* Immunity, 2003. **19**(1): p. 71-82.
314. Serbina, N.V. and E.G. Pamer, *Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2.* Nat Immunol, 2006. **7**(3): p. 311-7.

315. Serbina, N.V., et al., *TNF/iNOS-Producing Dendritic Cells Mediate Innate Immune Defense against Bacterial Infection.* Immunity, 2003. **19**(1): p. 59-70.
316. Kurihara, T., et al., *Defects in Macrophage Recruitment and Host Defense in Mice Lacking the CCR2 Chemokine Receptor.* J. Exp. Med., 1997. **186**(10): p. 1757-1762.
317. Mordue, D.G. and L.D. Sibley, *A novel population of Gr-1+-activated macrophages induced during acute toxoplasmosis.* J Leukoc Biol, 2003. **74**(6): p. 1015-1025.
318. Robben, P.M., et al., *Recruitment of Gr-1+ monocytes is essential for control of acute toxoplasmosis.* J Exp Med, 2005. **201**(11): p. 1761-9.
319. Sato, N., et al., *CC Chemokine Receptor (CCR)2 Is Required for Langerhans Cell Migration and Localization of T Helper Cell Type 1 (Th1)-inducing Dendritic Cells: Absence of CCR2 Shifts the Leishmania major-resistant Phenotype to a Susceptible State Dominated by Th2 Cytokines, B Cell Outgrowth, and Sustained Neutrophilic Inflammation.* J. Exp. Med., 2000. **192**(2): p. 205-218.
320. Nakano, H., et al., *Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses.* Nat Immunol, 2009. **10**(4): p. 394-402.
321. Ritter, U., et al., *TNF controls the infiltration of dendritic cells into the site of Leishmania major infection.* Medical Microbiology and Immunology, 2007. **July**.
322. Geissmann, F., et al., *Blood monocytes: distinct subsets, how they relate to dendritic cells, and their possible roles in the regulation of T-cell responses.* Immunol Cell Biol, 2008. **86**(5): p. 398-408.
323. Pasparakis, M., et al., *Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response.* J Exp Med, 1996. **184**(4): p. 1397-411.
324. Moore, T.A., et al., *Increased Mortality and Dysregulated Cytokine Production in Tumor Necrosis Factor Receptor 1-Deficient Mice following Systemic Klebsiella pneumoniae Infection.* Infect. Immun., 2003. **71**(9): p. 4891-4900.
325. Fremond, C., et al., *Membrane TNF confers protection to acute mycobacterial infection.* Respir Res. , 2005. **6**(1): p. 136.
326. Zganiacz, A., et al., *TNF-alpha is a critical negative regulator of type 1 immune activation during intracellular bacterial infection.* J Clin Invest, 2004. **113**(3): p. 401-13.
327. Grivennikov, S.I., et al., *Distinct and Nonredundant In Vivo Functions of TNF Produced by T Cells and Macrophages/Neutrophils: Protective and Deleterious Effects.* Immunity, 2005. **22**(1): p. 93-104.
328. Mauel, J., A. Ransijn, and Y. Buchmuller-Rouiller, *Killing of Leishmania parasites in activated murine macrophages is based on an L-arginine-dependent process that produces nitrogen derivatives.* J Leukoc Biol, 1991. **49**(1): p. 73-82.
329. Assreuy, J., et al., *Production of nitric oxide and superoxide by activated macrophages and killing of Leishmania major.* Eur J Immunol, 1994. **24**(3): p. 672-6.
330. Green, S.J., et al., *Activated macrophages destroy intracellular Leishmania major amastigotes by an L-arginine-dependent killing mechanism.* J Immunol, 1990. **144**(1): p. 278-283.

331. DomÌnguez, P.M. and C. ArdavÌn, *Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation.* Immunol Rev, 2010. **234**(1): p. 90-104.
332. Narni-Mancinelli, E., et al., *Memory CD8+ T cells mediate antibacterial immunity via CCL3 activation of TNF/ROI+ phagocytes.* J Exp Med, 2007. **204**(9): p. 2075-87.
333. Dunay, I.R., A. Fuchs, and L.D. Sibley, *Inflammatory Monocytes but Not Neutrophils Are Necessary To Control Infection with Toxoplasma gondii in Mice.* Infect. Immun. **78**(4): p. 1564-1570.
334. Brem-Exner, B.G., et al., *Macrophages Driven to a Novel State of Activation Have Anti-Inflammatory Properties in Mice.* J Immunol, 2008. **180**(1): p. 335-349.
335. Gabrilovich, D. and S. Nagaraj, *Myeloid-derived suppressor cells as regulators of the immune system.* Nat Rev Immunol, 2009. **9**(3): p. 162-74.
336. Cook, M.C., et al., *Generation of Splenic Follicular Structure and B Cell Movement in Tumor Necrosis Factor-deficient Mice.* J. Exp. Med., 1998. **188**(8): p. 1503-1510.
337. Ato, M., et al., *Loss of Dendritic Cell Migration and Impaired Resistance to Leishmania donovani Infection in Mice Deficient in CCL19 and CCL21.* J Immunol, 2006. **176**(9): p. 5486-5493.
338. Martin-Fontecha, A., et al., *Regulation of Dendritic Cell Migration to the Draining Lymph Node: Impact on T Lymphocyte Traffic and Priming.* J. Exp. Med., 2003. **198**(4): p. 615-621.
339. Sunderkotter, C., et al., *Subpopulations of Mouse Blood Monocytes Differ in Maturation Stage and Inflammatory Response.* J Immunol, 2004. **172**(7): p. 4410-4417.
340. Varol, C., et al., *Monocytes give rise to mucosal, but not splenic, conventional dendritic cells.* J Exp Med, 2007. **204**(1): p. 171-80.
341. Tacke, F. and G.J. Randolph, *Migratory fate and differentiation of blood monocyte subsets.* Immunobiology, 2006. **211**(6-8): p. 609-618.
342. Swirski, F.K., et al., *Ly-6Chi monocytes dominate hypercholesterolemia-associated monocytosis and give rise to macrophages in atheromata.* J Clin Invest, 2007. **117**(1): p. 195-205.
343. Mildner, A., et al., *Ly-6G+CCR2- Myeloid Cells Rather Than Ly-6ChighCCR2+ Monocytes Are Required for the Control of Bacterial Infection in the Central Nervous System.* J Immunol, 2008. **181**(4): p. 2713-2722.
344. Auffray, C., et al., *Monitoring of Blood Vessels and Tissues by a Population of Monocytes with Patrolling Behavior.* Science, 2007. **317**(5838): p. 666-670.
345. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* J Exp Med, 2007. **204**(12): p. 3037-47.
346. Fremond, C., et al., *Membrane TNF confers protection to acute mycobacterial infection.* Respiratory Research, 2005. **6**(1): p. 136.
347. Chu, C.Q., et al., *Localization of tumor necrosis factor alpha in synovial tissues and at the cartilage-pannus junction in patients with rheumatoid arthritis.* Arthritis Rheum, 1991. **34**(9): p. 1125-32.
348. Tracey, D., et al., *Tumor necrosis factor antagonist mechanisms of action: A comprehensive review.* Pharmacology & Therapeutics, 2008. **117**(2): p. 244-279.

349. Saxne, T., et al., *Detection of tumor necrosis factor alpha but not tumor necrosis factor beta in rheumatoid arthritis synovial fluid and serum.* Arthritis Rheum, 1988. **31**(8): p. 1041-5.
350. Elliot, M.J., et al., *Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha.* Arthritis Rheum, 2008. **58**(2 Suppl): p. S92-S101.
351. Baraliakos, X. and J. Braun, *Anti-TNF-alpha therapy with infliximab in spondyloarthritides.* Expert Rev Clin Immunol. **6**(1): p. 9-19.
352. Son, J.H. and S.W. Cha, *Anti-TNF-alpha Therapy for Ankylosing Spondylitis.* Clin Orthop Surg. **2**(1): p. 28-33.
353. Levin, A. and O. Shibolet, *Infliximab in ulcerative colitis.* Biologics, 2008. **2**(3): p. 379-88.
354. Molto, A., et al., *Visceral leishmaniasis and macrophagic activation syndrome in a patient with rheumatoid arthritis under treatment with adalimumab.* Joint Bone Spine, 2010. **77**(3): p. 271-273.
355. Kwiatkowski, D., et al., *Anti-TNF therapy inhibits fever in cerebral malaria.* Q J Med, 1993. **86**(2): p. 91-8.
356. Jacobs, P., D. Radzioch, and M.M. Stevenson, *In vivo regulation of nitric oxide production by tumor necrosis factor alpha and gamma interferon, but not by interleukin-4, during blood stage malaria in mice.* Infect Immun, 1996. **64**(1): p. 44-9.
357. Bean, A.G.D., et al., *Structural Deficiencies in Granuloma Formation in TNF Gene-Targeted Mice Underlie the Heightened Susceptibility to Aerosol Mycobacterium tuberculosis Infection, Which Is Not Compensated for by Lymphotoxin.* J Immunol, 1999. **162**(6): p. 3504-3511.
358. Ehlers, S., et al., *Lethal Granuloma Disintegration in Mycobacteria-Infected TNFRp55-/- Mice Is Dependent on T Cells and IL-12.* J Immunol, 2000. **165**(1): p. 483-492.
359. Murray, H.W., et al., *Visceral Leishmaniasis in Mice Devoid of Tumor Necrosis Factor and Response to Treatment.* Infect. Immun., 2000. **68**(11): p. 6289-6293.