Investigating the role of CD28 costimulation and IL-4/IL-13 responsive myeloid and lymphoid cells during helminth infections in mice

Hlumani H. Ndlovu

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Division of Immunology
Department of Clinical Laboratory Sciences
Faculty of Health Sciences
University of Cape Town
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PREFACE

The experimental work described in this thesis was carried out at the Division of Immunology, University of Cape Town, Observatory, from July 2009 to December 2012, under the supervision of Prof. Frank Brombacher and co-supervision of Dr. William Horsnell.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma at any University. Where use has been made of the work of others, it is duly acknowledged in the text.

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Hlumani H. Ndlovu (candidate)

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Prof. Frank Brombacher (supervisor)

.....

Dr. William Horsnell (co-supervisor)

DEDICATION

I dedicate this work to the women who brought me to this earth, my mother, Sibongile Veronica Ndlovu and to my son, Siyasanda Ntuthuko Ndlovu.

DECLARATION - PLAGIARISM

I, Hlumani Humphrey Ndlovu, hereby declare that

1. The research reported in this thesis, except where otherwise stated, is my original research.
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Hlumani H. Ndlovu

March 2013

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ABBREVIATIONS

aaMph's	-	Alternatively activated macrophages
ANOVA		Analysis of variance
AP	-	Alkaline phosphatase
APC	-	Allophycocyanin
APCs	-	Antigen presenting cells
AST		Aspartate transaminase
BCA	-	Bicinchoninic Acid Protein Estimation
Bio	-	Biotin
BM	-	Bone marrow
CAB		Chromotrope 2R and analine blue
CD		Cluster of differentiation
Cre	-	Cyclization recombinase
DCs	-	Dendritic cells
ELISA	-	Enzyme-linked Immunosorbent Assay
FACS	-	Fluorescent-activated cell sorter
FCS	-	Foetal calf serum
FITC	-	Fluorescein isothiocynate
FoxP3		Forkhead box P3
H&E	-	Haemotoxylin and eosin
HRPO	-	Horseradish peroxidase
IFN-γ	-	Interferon-gamma
Ig	-	Immunoglobulin
IL	-	Interleukin
IL-4Rα	-	Interleukin-4 receptor-alpha
IL-13Rα1	-	Interleukin-13 receptor-apha1
IL-13Rα2	-	Interleukin-13 receptor-alpha2
IMDM	-	Iscove's Modified Dulbecco's Medium

I.p	-	Intraperitoneal
L. major	-	*Leishmania major*
LN	-	Lymph node
mAbs	-	Monoclonal antibodies
MHC	-	Major histocompatibility complex
N.brasiliensis	-	*Nippostrongylus brasiliensis*
ND	-	Not detected
NO		Nitric oxide
NOS		Nitric oxide synthase
PAS	-	Periodic acid-Schiff reagent
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PE	-	Phycoerythrin
PMA	-	Phorbol 12-myristate 13-acetate
PNP	-	4-Nitrophenylphosphate
SD		Standard deviation
SEA	-	Soluble egg antigen
S. mansoni	-	*Schistosoma mansoni*
STAT	-	Signal transducer and activator of transcription
T-bet	-	T-box expressed in T cells
TCR	-	T cell receptor
Th	-	T helper
TLR	-	Toll-like receptors
TNF-α		Tumour necrosis factor alpha
Treg		Regulatory T cell
WT		Wild-type

PUBLICATIONS

1. **H. Ndlovu**, M. Darby, M. Froelich, W. Horsnell, F. Lühder T. Hünig and F. Brombacher. CD28 is required for protection against *Nippostrongylus brasiliensis* secondary infection and recall of memory responses in inducible CD28 deleting mice. *PlosPathogens*. Reviewed and under corrections.

2. WGC Horsnell, JC Hoving, M Darby, N Nieuwenhuizen, S Bobat, **H Ndlovu**, M Kimberg, F Kirsten, AJ Cutler, B DeWals, AF Cunningham and F Brombacher. Protective immunity against *Nippostrongylus brasiliensis* requires IL-13 production and antigen presentation by IL-4Rα responsive B cells. *PlosPathogens*. Reviewed and under corrections.

3. **H. Ndlovu**, N. Nieuwenhuizen, W. Horsnell, E. Hobeika , M. Reth and F. Brombacher. IL-4/IL-13 responsive B cells are required for protection against acute schistosomiasis and down-modulating liver and gut inflammation. Manuscript prepared.

4. **H. Ndlovu**, N. Niuweinhuizen, W. Horsnell, and F. Brombacher. IL-4/IL-13 responsive $CD11c^{+}$ dendritic cells are required for protection against acute Schistosomiasis and modulate T helper cell immunity. Manuscript prepared.

Abstract

ABSTRACT

IL-13 is a key cytokine orchestrating the development of protective immune responses against *Nippostrongylus brasiliensis* (*N. brasiliensis*) infection. The contribution of CD28 costimulation during primary responses to helminth infections is well established. Mice lacking CD28 expression failed to clear *N. brasiliensis* infection and exhibited impaired Th2 cytokine responses. The necessity of CD28 costimulation during recall of protective memory responses to pathogens has remained largely controversial. Hence, in this study, we investigated the role of CD28 during recall of memory responses to *N. brasiliensis* infection using a novel mouse model that allows for *cd28* gene deletion by oral administration of estrogen receptor analogue tamoxifen. As expected, $CD28^{-/-}$ mice failed to clear primary *N. brasiliensis* infection due to impaired cellular and humoral immune responses. Interestingly, deletion of *cd28* gene by oral administration of tamoxifen in $CD28^{-/lox}Cre^{+/-}$ mice prior to secondary infection resulted in abrogated worm expulsion, reduced recruitment of $CXCR5^{+}$ T_{FH} cells, reduced expansion of central and effector memory $CD4^{+}$ T cells and follicular B cells. Likewise, $CD28^{-/-}$ mice failed to expel *N. brasiliensis* worms during secondary infection and displayed reduced numbers of $CXCR5^{+}$ T_{FH} cells. Together, these data demonstrate a crucial role for CD28 costimulation in recall of protective Th2 immune responses and expansion of $CXCR5^{+}$ T_{FH} cells during *N. brasiliensis* secondary infection.

Protective immunity to *S. mansoni* infection is dependent on the development of sufficient $CD4^{+}$ T cell driven Th2 immune response and granuloma formation. Mice deficient in IL-4, IL-4/IL-13, IL-4/IL-10, IL-4Rα and signal transducer and activator of transcription-6 (STAT-6) were found to be highly susceptible to *S. mansoni* infection due to impaired Th2 polarisation, reduced granuloma formation, reduced fibrosis and increased tissue inflammation. Studies from our laboratory using cell-specific IL-4Rα deficient mice have been crucial in elucidating mechanisms conferring protection or susceptibility to acute Schistosomiasis. Mice lacking IL-4/IL-13 responsive macrophages and neutrophils ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$) quickly succumbed to *S. mansoni* infection compared to littermate control mice despite the presence of Th2 cytokines. Heightened susceptibility in $LysM^{cre}IL\text{-}4R\alpha^{-/lox}$ mice was associated with augmented liver pathology and destruction of gut integrity that ultimately led to endotoxemia and septic shock. IL-4Rα expression on pan-T cells and smooth muscle cells is crucial for protection against acute schistosomiasis.

In this study, we investigated the role of IL-4Rα expression on B cells using mice deficient in IL-4Rα expression specifically on B cells (*mb1*^{cre}IL-4Rα$^{-/lox}$) and on CD11c^{+} DC's and alveolar macrophages by utilising CD11c^{cre}IL-4Rα$^{-/lox}$ mice. *Mb1*^{cre}IL-4Rα$^{-/lox}$ mice were highly susceptible to *S. mansoni* infection compared to littermate control mice due to impaired production of Th2 cytokines, augmented granuloma formation, exacerbated hepatocellular damage and increased gut inflammation. Furthermore, the absence of IL-4/IL-13 responsive B cells resulted in abrogated intracellular cytokine secretion indicated by reduced production of both Th1 and Th2 cytokines by CD4^{+} T cells and CD19^{+} B cells. More importantly, mice deficient in IL-4Rα expression on B cells failed to down-regulate granuloma formation and CD4^{+} T cells responsiveness indicated by uncontrolled Th1, Th2 and Th17 cytokines production during the chronic stages of infection. Lastly, we investigated whether IL-4 derived from B cells drives host protective immune responses during *S. mansoni* infection in mice. Since there are no transgenic mice that restrict IL-4 expression specifically on B cells, we exploited bone marrow chimeras. Infection of chimeras lacking IL-4 derived from B cells showed that IL-4 is not a key cytokine initiating host protective immune responses, as demonstrated by unaltered humoral immunity, reduced granuloma formation and sufficient cytokine production. Therefore, these data demonstrates that the ability of B cells to respond to Th2 stimuli provided by IL-4/IL-13 but not their capacity to produce IL-4 is crucial for development of host protective immune responses during *S. mansoni* infection in mice.

CD11c^{cre}IL-4Rα$^{-/lox}$ mice succumbed to *S. mansoni* infection and presented with body weight loss at the chronic stages of infection compared to littermate control mice. Death was caused by elevated serum levels of pro-inflammatory cytokine TNF-α and colitis-type pathology despite controlled liver and gut inflammation. Interfering with IL-4Rα expression on DCs and alveolar macrophages resulted in impaired CD4^{+} T cell activation, reduced intracellular cytokine secretion of IL-4, IL-5 and IFN-γ by CD4^{+} T cells and classical activation of DCs and macrophages indicated by increased iNOS expression. Together, these data demonstrate that IL-4/IL-13 responsive DC's and alveolar macrophages are essential for protection against *S. mansoni* infection and are involved in initiating and amplifying Th1 and Th2 immune responses.

CHAPTER 1

INTRODUCTION

1.0 Immune System

The immune system is a body's defence mechanism against foreign invading pathogens such as viruses, bacteria and parasites [1]. The immune system has evolved the capacity to specifically recognise and eliminate pathogens and foreign particles (antigens) whilst exercising tolerance towards "self" antigens [1]. The immune system comprises of two parts; namely innate (natural) immunity that arises from primary exposure to pathogens and adaptive (acquired) immunity that is a more complex, and highly specific system arising from infection [2,3].

1.1 Innate Immunity

Innate immunity is considered to be the "first line defence" against invading pathogens [1]. Innate immunity consists of cells such as neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, basophils, mast cells, platelets and the complement system. Innate cells such as macrophages and neutrophils mediate host protection by internalising (phagocytosis) and directly killing pathogens, a process that involves recognition of a wide array of conserved structural moieties on pathogen surfaces known as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) [4,5,6,7]. Importantly, binding of PAMPS to PRRs is a cornerstone to innate immunity, leading to activation of the complement system and uptake and processing of antigen by antigen presenting cells (APCs) like dendritic cells, monocytes and macrophages [1]. Complement and C-reactive proteins are important serum proteins that bind to pathogen membranes, mediating their killing [1,8,9].

Eosinophils are involved in attacking pathogens that are too large for phagocytosis by releasing cytotoxic factors like eosinophil peroxidase (ESO) [10]. Furthermore, they limit inflammation by releasing enzymes that inhibit the activity of mast cell products [4]. Natural killer cells are involved in killing cells that are infected with intracellular pathogens. Studies have shown that NK cells initiate the immune response by producing IFN-γ, a pro-inflammatory cytokine [11], while NK T cells produce IL-4 in innate immunity [12].

1.2 Adaptive immunity

Adaptive immunity is a highly specific arm of the immune system that is co-ordinated to ensure a response that is appropriate in quality and magnitude to the eliciting antigenic stimuli. A key feature of adaptive immunity is development of immunological "memory responses" that ensures immediate recognition of pathogens during re-infection [1]. It is orchestrated by B and T lymphocytes, which express randomly generated antigenic receptors that possess the ability to recognise a wide array of antigens. Antigens are presented to lymphocytes by APCs, which provide an interface between innate and adaptive immunity [6]. APCs carrying antigen on major histocompatibility class I molecule (MHC I) activate cytotoxic $CD8^+$ T cells that are involved in killing of cell infected with intracellular pathogens [1] Conversely, $CD4^+$ T helper (Th) cells receive antigenic stimuli via presentation by MHC II molecules on the surface of APCs. $CD4^+$ T cells mediate immunity against extracellular pathogens, allergens, viruses and bacterial infections.

1.2.1 Cytokines

Cytokines are small extracellular proteins that act as messenger molecules during host defences (immunity) and are involved in tissue repair, cell division and differentiation. Cytokines can be produced and secreted by a variety of cells in response to diverse stimuli. A cell possesses the capacity to produce a wide array of cytokines from its repertoire. However, the type of cytokine the cell makes is dependent on the activating stimuli, its nature, duration, intensity as well as presence of other factors like hormones and cellular interactions [13]. Cytokines are pleiotropic in a short operational radius and they can act directly on cells producing them (autocrine) or on other surrounding cells (paracrine) ([1,14]. The production of cytokines is transient under normal circumstances but it can be prolonged during a disease state [15,16]. The function of cytokines has been studied by utilising gene knock-out mice. However, a caveat for using this system is that absence of a gene during thymic development/ontogeny may result in compensatory mechanisms that may not be present in gene sufficient mice [17].

Cytokines are grouped into families based on common structural features. A summary of cytokine families is provided in Table 1.1 below.

Table 1.1: Summary of cytokine families grouped based on structural similarities of ligands and/or receptors. Adapted from [13].

Family	Abbreviation	Name
Hematopoietins	IL-2	Interleukin-2
	IL-4	Interleukin-4
	IL-5	Interleukin-5
	IL-6	Interleukin-6
	IL-10	Interleukin-10
	IL-12	Interleukin-12
	GM-CSF	Granulocyte macrophage colony stimulating factor
	G-CSF	Granulocyte colony stimulating factor
TNF family	TNFα	Tumor necrosis factor α
	LTα/β	Lymphotoxin α or β
IL-1 family	IL-1α	Interleukin-1 α
	IL-1β	Interleukin-1 β
α (CXC) family	IL-8	Interleukin-8
	NAP-2	Neutrophil activating protein 2
β (CC) family	MCP1/2/3	Monocyte chemoattractant protein 1/2/3
	MIP 1α/β	Macrophage inflammatory protein 1α/β
	RANTES	Regulated upon activation normal T cell expressed and secreted
PDGF family	PDGF A/B	Platelet-derived growth factor A/B
	CSF	Macrophage colony-stimulating factor
	SCF	Stem cell factor
TGFβ family	TGFβ	Transforming growth factor β

1.2.2 $CD4^+$ T cell differentiation

Differentiation of naive $CD4^+$ T cells is dependent on type of APC, co-stimulatory molecules, the nature and doses of antigen and more importantly, the cytokine microenvironment that T cell experiences during antigen presentation [3,18,19]. Traditionally, $CD4^+$ T cells were subdivided into Th1 or Th2 subsets based on their cytokine secretion profiles [20,21,22,23]. However, recent

studies have extended the number of $CD4^+$ T cell subsets to include Th17 [24,25,26], Tregs [27,28], Th9 [29] and Th5 [30]. The T cell paradigm is illustrated in Figure 1.1.

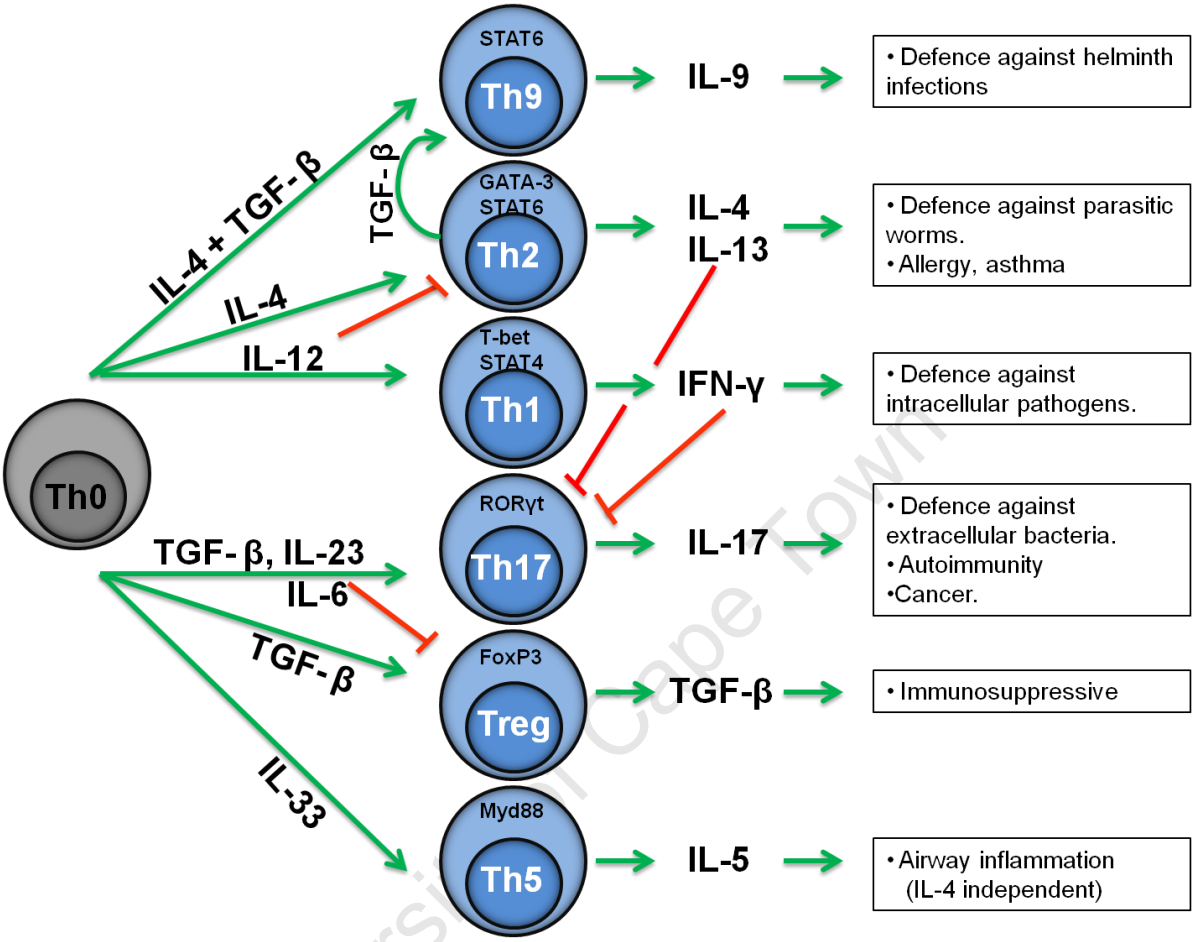

Figure 1.1: T-helper cell differentiation.
Naive $CD4^+$ T cells differentiate into Th1, Th2, Th5, Th9, Th17 and Tregs upon receiving antigenic stimulation in the presence of cytokines. Th1 differentiation is governed by IL-12 (from DCs) and IL-18 while Th2 is prompted by IL-4 produced by NK, B cells, basophils and γδ cells. Th9 cell differentiation is orchestrated by TGF-β in association with IL-4. However, Th2 cells can be reprogrammed into Th9 cells in the presence of TGF-β. IL-23 and IL-6 are important for differentiation of Th17 cells during inflammatory responses while IL-33 drives the differentiaton of Th5 cells. Regulatory T cells produce an immunosuppressive cytokine IL-10 after stimulation with TGF-β. Red arrows indicate cross-regulatory effects. The illustration is adapted from previous publications ([14,25,29].

Differentiation of Th1 cells is triggered by IL-12 signalling via a STAT-4 dependent pathway and induces the production of the hallmark cytokines IFN-γ and TNF-α [31]. STAT-4 signalling up-regulates the transcription factor T-box expressed in T cells (T-bet) [32,33,34], which in turn promotes IFN-γ and IL-12R (IL-12Rβ2) expression, thus creating a positive feedback mechanism [35]. Th1 cells mediate protection against intracellular pathogens by mediating antibody responses, activating macrophages and delayed-type hypersensitivity. In contrast, Th2 immunity driven by IL-4 signalling is crucial for protection against helminth infections [36,37,38,39] and

exacerbates allergies and asthma ([40,41,42]. Th2 cell differentiation occurs upon engagement of T cell receptor (TCR) and IL-4Rα signalling via STAT-6 pathway that up-regulates the expression of the transcription factor GATA-3 [43,44]. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13. IFN-γ produced by Th1 cells counter-regulate the development of the Th2 immune response while IL-4 down-regulates the Th1 immune response [45]. Furthermore, STAT-6 has been shown to suppress Th1 development by inhibiting IL-12Rβ2 expression while STAT-4 prevents GATA-3 expression by Th2 cells [35].

Th17 cells produce IL-17 after receiving antigenic stimulation in the presence of IL-6, TGF-β and IL-23 [24,26]. Differentiation of Th17 cells is directed by the orphan nuclear receptor (RORγt) [46]. These cells are important for expulsion of extracellular microbes [47], autoimmunity [48] and cause asthmatic symptoms [49]. Tregs stimulated by TGF-β signalling are identified by the expression of CD25 and the transcription factor forkhead box P3 (FoxP3) [27]. IL-6 can negatively regulate the generation of Tregs, cells that are crucial for suppressing immunity towards "self" and "non-self" antigens [28]. Recently, TGF-β has been shown to be capable of reprogramming Th2 cells into IL-9 producing Th9 cells in the presence of IL-4 [29]. Lastly, IL-33 signalling via ST2 and Myd88 pathways induces the differentiation of IL-5 secreting Th5 cells independently of IL-4 or STAT-6 [30,50].

1.2.2.1 T cell activation

Differentiation of naive T cells requires three signals; the first provided by the interaction between the T cell receptor (TCR) and the antigen presenting MHC molecule, secondly the interaction of the costimulatory molecule CD28 with it ligands CD80 and CD86 and lastly, secretion of polarising cytokines (Figure 1.2) [51,52]. Studies have demonstrated a crucial role for CD28 costimulation in $CD4^+$ T cell activation [53,54], organisation of secondary lymphoid tissue [54,55,56] and production of antigen-specific antibodies during *S. mansoni* infection [57] and *Leishmania major* infection [58]. Abrogation of CD28 costimulation results in impaired recruitment of follicular helper T cells (T_{FH}); essential for development of germinal centers, isotype switching and differentiation of plasma cells through secreting their effector molecule IL-21 [59,60,61,62].

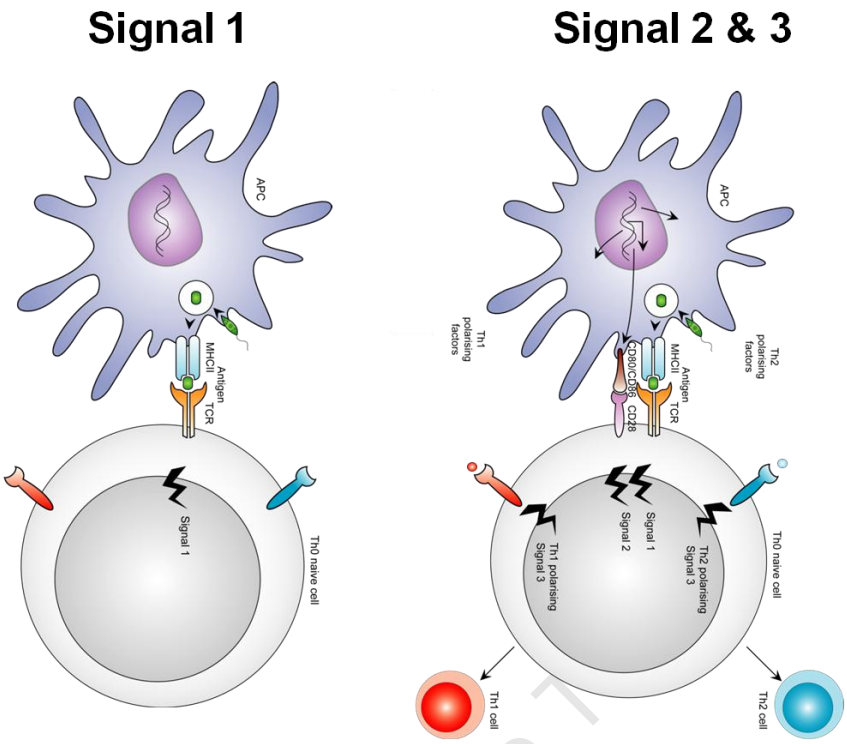

Figure 1.2: T cell activation.
Activation of T cells requires three signals provided by the interaction between the TCR and MHC molecule, CD28 binding to its ligands CD80/CD86 and secretion of cytokines. T cells receiving only signal 1 becomes unresponsive and undergo apoptosis. This figure was adapted from [63,64,65].

The contribution of CD28 costimulation in protective immunity to primary infection with helminths is well established. Infection of $CD28^{-/-}$ with *S. mansoni* [57] and *Nippostrongylus brasiliensis* [66,67] showed that CD28 is essential for development of protective Th2 immunity. However, the contribution of CD28 in recall of memory responses has remained largely controversial. Harris and colleagues showed that CD28 is not required for recall of memory T cell responses during *N. brasiliensis* infection [66]. Consistent with this data, infection of $CD28^{-/-}$ mice with *H. polygyrus* and *B. dermatitidis* demonstrated that recall of memory responses occurs independently of CD28 costimulation [68,69]. Conversely, development of memory responses during *T. gondii* infection was found to be CD28 dependent [70]. Likewise, clearance of persistent viral infection is dependent on CD28 costimulation [71,72]. Hence, more studies are required to clarify the role of CD28 during recall of memory responses to helminth infection through using appropriate tools and infectious models.

1.2.3 B cell differentiation

B cells originate and begin their development process in the bone marrow (BM). Early events in B cell development involve the error-prone process of combinatorial rearrangement of the H chain locus and the L chain locus [73,74]. Studies in mice have found that surrogate L chain (SLC) proteins are crucial regulatory proteins during early B cells development stages [75]. The SLC proteins pair up with the μ H chain to form pre-B cell receptor (pre-BCR) in murine B cells [76]. Progenitor B cells (pro-B) that do not express pre-BCR or surface immunoglobulin (Ig) develop into pre-B cells (Figure 1.3). B cells complete their maturation and development process outside the BM. Pre-B cells exiting the BM acquire surface expression of IgD and CD21 and develop into immature B cells that are subdivided into transitional 1 (T1) or T2 based on phenotype and ontogeny (Figure 1.3). Interestingly, immature B cells are capable of producing rapid antibody responses independently of MHC class II-restricted T-cell help during exposure to T cell-independent antigens like lipopolysaccharides (LPS) [77].

B cells have been classified into two populations; B-1 cells that express CD5 and B-2 cells that are CD5 negative. B-1 cells are distinguished from B-2 cells by anatomic localisation, self-renewal and capacity to secrete natural antibodies [78]. B-1 cells are further subdivided into B-1a ($CD5^+$) and B-1b ($CD5^-$) (Figure 1.3) and they have unique functions within the host. B-1a cells produce natural antibodies that provide innate protection against bacterial infection in naive hosts, while B-1b cells secrete long-term adaptive antibody responses to T cell independent antigens during infection [79]. B-2 cells consists of conventional B cells that upon receiving antigenic stimuli via the interaction of the BCR and antigen carrying follicular DCs and expression of Notch proteins, proliferate and differentiate into either non-circulating cells that populate the marginal zone (MZ) or into circulating cells that form the germinal center (GC) [80]. MZ B cells differentiate into plasma cells (PCs) that produce low affinity IgM antibodies [81,82] and provide defence against blood-borne bacteria [77].

Mature B cells residing in the lymphoid follicles of the spleen and lymph nodes receive antigenic stimulation via the BCR and become activated, leading to formation of the GC, transient generation of plasmablasts, and short-lived extrafollicular plasma cells that secrete antigen specific antibodies (Figure 1.3). Memory B cells develop within the GC through somatic hypermutation (SMH) of the V_H genes, class switch recombination (CSR) and affinity maturation of the BCR for a specific antigenic epitope [83,84]. Memory B cells respond rapidly and secrete

high affinity antibodies during secondary exposure to antigen by differentiating into PCs [85]. Development of PCs is tightly regulated by transcription factor Bcl-6 and BLIMP-1 [86].

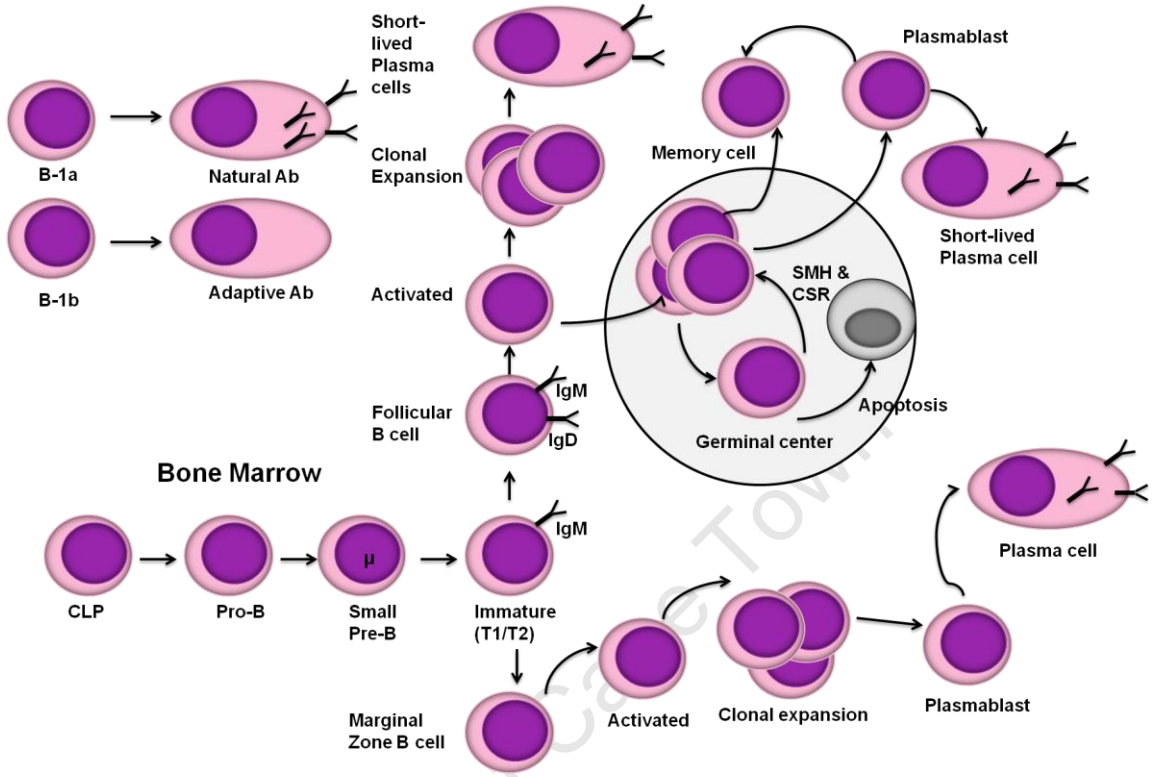

Figure 1.3: B cell development
B cells are generated in the bone marrow. B cells develop from a common lymphocyte progenitor (CLP) and develop into various B cell subsets upon rearrangement of the immunoglobulin H and L chains, expression and engagement of B cell receptor (BCR) and somatic hypermutation. The figure was adapted from [77].

1.2.3.2 Functions of B cells in immunity

Traditionally, B cells are known for their ability to produce antigen-specific antibodies that facilitate clearance of pathogens and other antigens. However, the scope of B cells in the immune response to pathogens has been expanded over the years. B cells play a crucial role in the priming and proliferation of $CD4^+$ T cells after encounter with antigen [87,88,89]. Chimeric reporter mice deficient of B cells had a significantly reduced number of IL-4 producing T cells compared to control mice following infection with *H. polygyrus*, suggesting that B cells are required for initiation and expansion of $CD4^+$ T cell responses during infection [90]. Consistent with this data, mice lacking B cells during lymphocytic choriomenigitis virus (LCMV) had reduced number of LCMV-specific IFN-γ producing $CD4^+$ T cells at day 8 and 70 post-infection [91]. Activated B cells have been shown to act as excellent antigen-presenting cells (APCs) capable of internalising specific antigen through the BCR and presenting antigen to $CD4^+$ T cells [92,93]. The ability of B

cells to present antigens is spectacularly demonstrated when antigen load is low and there is evidence suggesting that antigen-presenting B cells shape the magnitude and quality of the primary and memory $CD4^+$ T cell responses [89,94,95,96,97]. B cells deficient of co-stimulatory molecules CD80 and CD86 failed to prime and promote proliferation of autoreactive $CD4^+$ T cells during a mouse model of arthritis [98], thus demonstrating that B cells expressing co-stimulatory molecules modulate T cell responses [87,99]. B cells play a crucial role during lymphoid tissue organogenesis [100], formation of B cell follicles [101] and differentiation of follicular DCs [102,103]. Interestingly, B cells shape the quality and quantity of the immune response by producing a wide array of cytokines [93,104,105,106,107]. This is discussed in more details below.

1.2.3.1 B effector cells

B cells produce cytokines and chemokines constitutively or in response to antigenic stimulation, engagement of toll-like receptor (TLR) ligand and interaction with T cells [93,107]. Cytokines produced by B cells can amplify or suppress $CD4^+$ T cell responses. In a study by Shah and Qiao, resting splenic B cells co-cultured with T cells stimulated with CD3-specific antibody primed the expansion of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) [108]. The expansion of Tregs was mediated by TGF-β produced by splenic B cells [108]. Likewise, TGF-β produced by B cells promoted the conversion of $CD4^+CD25^-$ T cells into $FoxP3^+$ Tregs *in vivo* in a model of local inhalation tolerance [109].

Harris and colleagues identified two subsets of B effector cells based on their cytokine secretion profile; namely B effector 1 (Be1) and Be2 [106]. Be1 cells primed in the presence of Th1 cells, antigen and/or TLR ligand produced IFN-γ and IL-12p40 (Figure 1.4) [104,106]. The expansion of Be1 cells required signalling via the IFN-γR which activated the transcription factor T-box expressed in T cells (T-bet) [104]. In contrast, Be2 cells behaved similarly to Th2 cells by producing Th2 cytokines IL-2, IL-4 and IL-13 while simultaneously suppressing the secretion of IFN-γ and IL-12 after co-culture in the presence of Th2 cells and antigen (Figure 1.4) [105,106]. Importantly, signalling via the IL-4Rα mediates the proliferation of Be2 cells [105]. Effector molecules produced by Be1/Be2 cells can prime naive $CD4^+$ T cells, pushing them towards either Th1 or Th2 phenotype (Figure 1.4). Thus, interaction between effector B cells and Th1 or Th2

cells creates a positive feedback loop that ensures maintenance of the immune response during infection.

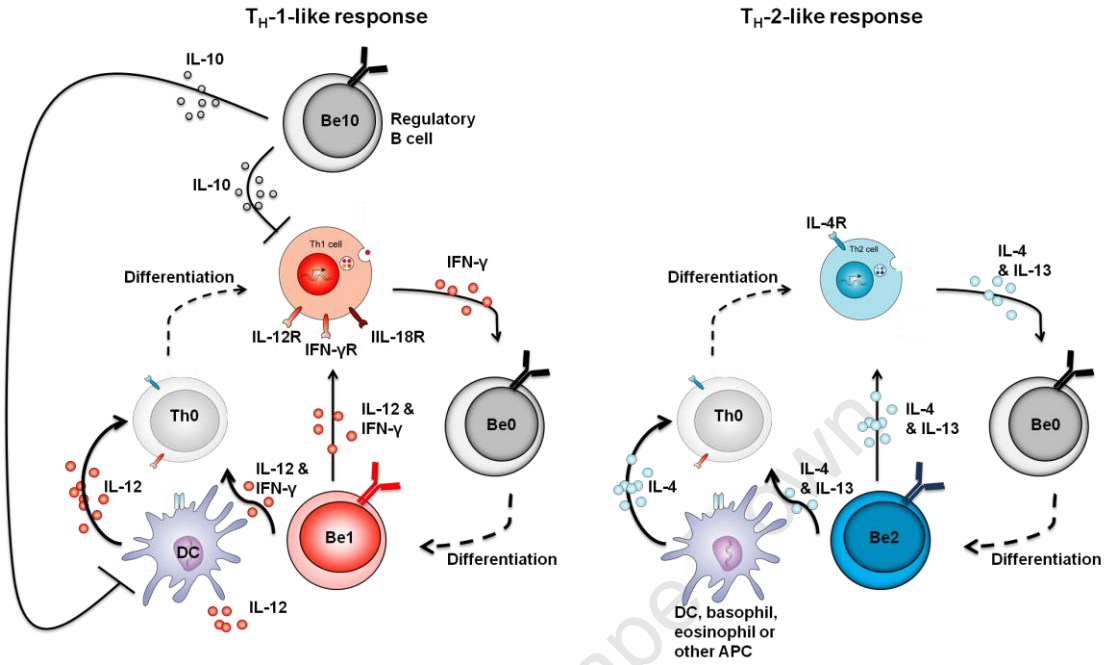

Figure 1.4: Effector and regulatory B cells enhance or suppress CD4$^+$ T cell responses.
B cells can influence activation of CD4$^+$ T cells by presenting antigen and providing costimulation and producing cytokines. B effector 1 cells (Be1) generated in the presence of Th1 cells and antigen produce predominantly Th1 cytokines like IL-12 and IFN-γ. In turn, cytokines produce by Be1 cells can activating naive T cells, pushing them towards a Th1 phenotype. Likewise, Be2 cells develop in the presence of Th2 cells and antigen and produce IL-4, IL-5 and IL-13. They could also influence the activation of naive T cells through the cytokines they produce. Regulatory B cells produce IL-10 and play a crucial role in regulating CD4$^+$ T cells responsiveness. The figure was adapted from [110].

Recently, a third subset of effector B cells that produces IL-10 (B10) was phenotypically and functionally characterised in mice during inflammatory responses [111]. IL-10 producing regulatory B cells were identified by a unique expression of CD1d and CD5 [111]. B10 cells stimulated with CD40L [112] and TLR ligand [113] suppress CD4$^+$ T cell responses and played a crucial role in down-regulating autoimmune pathology [114,115,116]. Furthermore, B10 cells have been shown to mediate protection against allergic airway inflammation [117], prevent colitis [118,119,120] and collagen-induced arthritis [121,122].

1.3 Interleukin 4/ IL-13

1.3.1 Interleukin-4

IL-4 belongs to the same cytokine family as IL-5, IL-13 and granulocyte macrophage-colony stimulating factor (GM-CSF) and their genes are located in close proximity on chromosome 11 [123]. Murine IL-4 is a glycoprotein with a molecular weight of 14-19 kDa depending on a cellular source secreting it [124,125]. IL-4 is secreted by various innate cells including basophils [126], mast cells [127], γδ T cells [128], conventional T cells [129,130], eosinophils [131] and $NK1.1^+$ T cells [12]. Although it is widely understood that IL-4 is essential for Th2 cells differentiation, some studies utilising IL-4Rα and STAT-6 deficient mice demonstrated that Th2 differentiation can occur independently of IL-4 signalling [132,133,134,135]).

The biological activities of IL-4 are species-specific; murine IL-4 is inactive on human cells and vice versa. IL-4 has a profound effect on B cell activation status by influencing the expression of low affinity IgE receptor [136], increasing expression of activation markers MHC II, CD80 and CD86 [137], up-regulating IL-4Rα expression [138] and promotes isotype switching to IgE and IgG1 while suppressing synthesis of IgM, IgG2a and IgG2b [139,140]. IL-4 is crucial for conferring host protection against gastrointestinal nematodes like *Trichuris muris* (*T. muris*) [141], *Heligomosomoides polygyrus* (*H. polygyrus*) [142,143] and *Nippostrongylus brasiliensis* (*N. brasiliensis*) [144,145]. Mice deficient in IL-4, IL-4Rα and STAT-6 were found to be highly susceptible to *Schistosoma mansoni* (*S. mansoni*) infection due to uncontrolled liver and intestinal inflammation ([146,147,148,149].

1.3.2 Interleukin-13

IL-13 is derived from a wide variety of cell types that includes T cells, DCs, basophils, NK cells and mast cells [150,151,152]. IL-13 gene is located on chromosome 11 between IL-4 and GM-CSF genes, and is translated into a 10-14 kDa immunoregulatory protein [153,154]. IL-13 shares some but not all biological activities with IL-4 [155]. The importance of IL-13 in driving worm expulsion was first demonstrated in studies with *N. brasiliensis* using IL-4 and IL-13 knock-out (KO) mice [145,156,157]. Furthermore, IL-13 has been shown to regulate pulmonary inflammation induced by allergens ([158,159,160], asthma [158,159,160,161,162,163] and anaphylaxis [146]. IL-13 exerts its effector function on a wide variety of cells such as fibroblasts,

B cells, macrophages/monocytes, mast cells, eosinophils, smooth muscle cells and vascular endothelium [164]. It is important to mention that IL-13 cannot act on T cells [165]. Similarly to IL-4, IL-13 is involved in the proliferation of B cells and up-regulates the expression of low affinity IgE receptor and induces isotype switching to IgG4 and IgE in human B cells [166]. IL-13 enhances the expression of integrins like CD11b and CD11c on monocytes and macrophages [165].

1.3.3 IL-4 and IL-13 receptor complexes

IL-4 and IL-13 signals through a common receptor subunit, the IL-4 receptor alpha chain (IL-4Rα) which might explain why they have overlapping function [132,167]. IL-4Rα is widely expressed on haematopoietic cells, endothelial, epithelial, muscle, fibroblasts, hepatocytes and brain tissue [167]. IL-4 uniquely signals through the heterodimeric type 1 receptor composed of IL-4Rα subunit and the common gamma chain (γc) [167,168], which is also a component of the receptor for IL-2, IL-7, IL-9, IL-15 and IL-21 [153,169]. However, both IL-4 and IL-13 signals through the type II receptor consisting of IL-4Rα and IL-13Rα1 subunits [167,168]. Receptors mediating IL-4 and IL-13 signalling are illustrated in Figure 1.5.

IL-13 specifically binds to the IL-13Rα1 with low affinity. However, the IL-13Rα1 and IL-4Rα complex binds IL-13 with high affinity and activates down-stream signalling molecules [153,170]. Another high affinity receptor for IL-13 is the homodimeric IL-13Rα2 that was initially thought to be a decoy receptor with no signalling abilities [171]. However, evidence has emerged showing that IL-13Rα2 is capable of inducing TGF-β production or mediating fibrosis in a chronic model of Crohn's disease [172,173]. IL-13 receptors are expressed in non-haematopoietic and haematopoietic cells except for T cells [153,174]. The activity of IL-4 and IL-13 can be regulated by binding to high affinity soluble IL-4Rα and IL-13Rα2 that carry no signalling abilities [175].

1.3.3 Mechanism of IL-4 and IL-13 signalling

Engagement of IL-4Rα triggers the activation of STAT-JAK signalling pathway [167]. Briefly, IL-4 binding to IL-4Rα induces the activation of JAK1 and 3 and phosphorylation of a specific tyrosine residue in the receptor cytoplasmic region. STAT-6 binds to the phosphorylated receptor

through a highly conserved domain and allows for phosphorylation of the C-terminal domain by the activated kinases [176,177]. Activated STAT-6 disengages from the receptor and forms homodimers through the interaction of the conserved domain with the phosphorylated C-terminal domain. The STAT-6 homodimers translocate into the nucleus where they bind to specific DNA motifs in the promoter of responsive genes thus enhancing transcription. IL-4 signalling via IL-4Rα has been shown to trigger the insulin receptor substrate (IRS) signalling pathway [178,179] and the phosphoinositide-3-kinase (PI-3-K) pathway [180,181]. IL-13 signalling through the IL-13Rα2 activates AP-1 and is involved in the production of TGF-β and fibrosis [172].

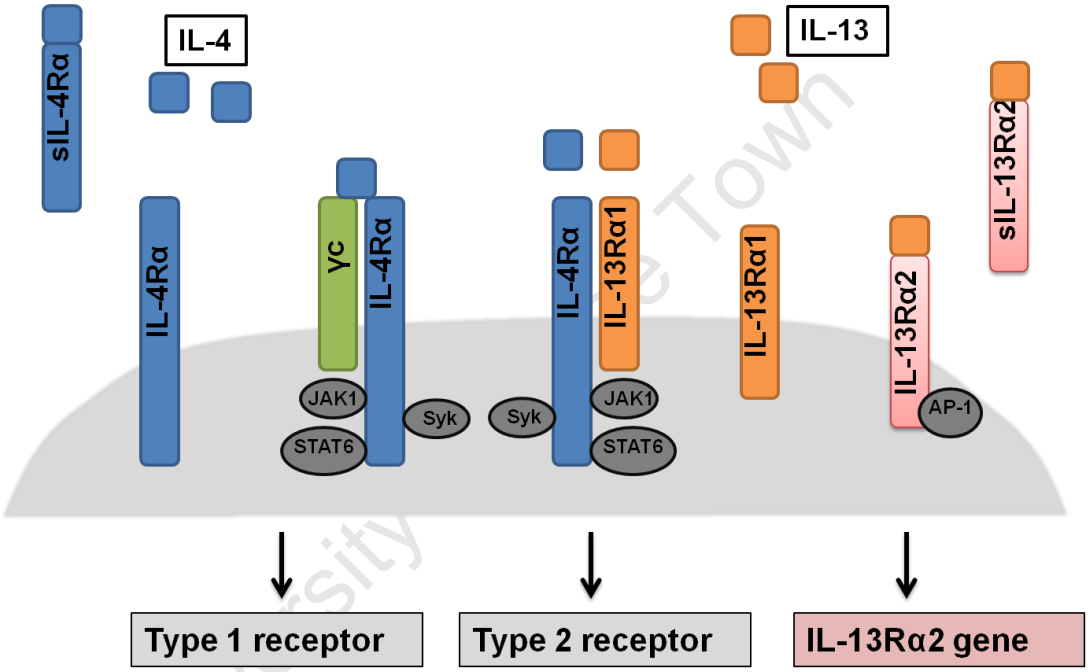

Figure 1.5: IL-4 and IL-13 receptor complexes.
IL-4 signals through a type 1 receptor composed of IL-4Rα subunit and γc while both IL-4 and IL-13 signals via a type II receptor consisting of IL-4Rα and IL-13Rα1 subunits. Binding of IL-4 and IL-13 to the receptors results in the phosphorylation of down-stream signalling molecules like signal transducer and activator of transcription 6 (STAT-6) and Janus kinase 1 (JAK1). IL-13 binds to the IL-13Rα2 with high affinity and activates AP-1 to induce the signalling cascade that results in TGF-β secretion. This figure is adapted from previous publications [14,153,155].

1.3.5 Cell-specific IL-4Rα gene targeting

Initially, the role of IL-4Rα signalling in host protection or susceptibility to pathogenic infections was studied using gene-deficient mice. However, the advent of Cre/*lox*P recombination system has allowed for cell-specific gene deletion [182,183,184]. Here, a transgenic mouse carrying the Cyclization recombinase (Cre) under the control of a cell-specific promoter is intercrossed with a transgenic mouse carrying the gene of interest flanked by a pair of *lox*P binding sites (Figure 1.6).

Once the promoter induces the expression of Cre recombinase in a specific cell-type, the enzyme carries our gene deletion by homologous recombination of the *lox*P sites [185]. To target cell-specific IL-4Rα gene deletion, a transgenic mouse carrying exon 7 to exon 9 of IL-4Rα gene flanked by *lox*P sites was generated. In our laboratory we have generated a transgenic mouse strain carrying cell-specific deletion of IL-4Rα on $CD4^+$ T cells [186,187], on macrophages and neutrophils [147], on smooth muscle cells [188,189]), on pan-T cells [190], on B cells [191] and on $CD11c^+$ DCs [192].

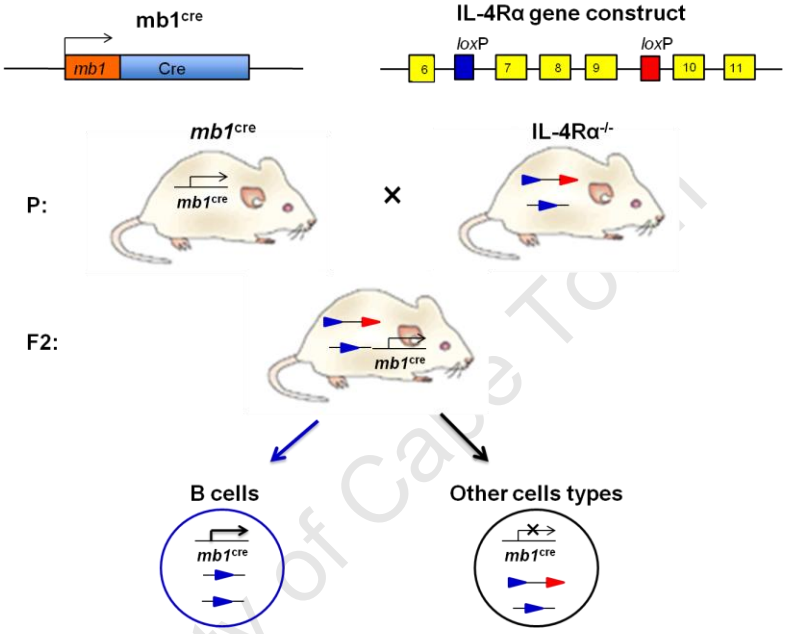

Figure 1.6: Generation of B cell-specific IL-4R deficient mice (*mb1*^{cre}IL-4Rα$^{-/lox}$).
IL-4Rα gene construct was created by flanking exon 7-9 with two *lox*P sites (blue and red colour) and Cre recombinase gene construct has *mb1* promoter down-stream *Cre* gene. IL-4Rα$^{-/-}$ mice were mated with *mb1* mice to generate the *mb1*^{cre}IL-4Rα$^{-/-}$ F1 offspring. This offspring was again mated with IL-4Rα$^{lox/lox}$ mice to generate *mb1*^{cre}IL-4Rα$^{-/lox}$ mice where *Cre* mediated deletion of IL-4Rα specifically on B cells. Figure is adapted from [190].

1.4 *Nippostrongylus brasiliensis*

Intestinal nematodes are the most prevalent infection in humans and are thought to infect approximately one billion people worldwide, particularly in developing regions of Asia, Africa and Latin America [38,193]. They are a major cause of under-nutrition and anaemia, thus have a negative impact on socio-economic development in areas of high prevalence [193]. Parasites belonging to the family *Strongylata* can be subdivided into three superfamilies; namely *Strongyloidea* consisting of parasites infective to humans (i.e *Necator americanus*), *Trichostronglyloidea* comprised of parasites infective to rodents (i.e *Nippostrongylus brasiliensis*) and *Metastrongyloidea* consisting lung infective worms in mammals. The infection and migration patterns of *N. brasiliensis* is analogous to human hookworms such as *N. americanus* and

Ancylostoma duodenale; thus, it can be used as a murine model to investigate the immune response to these parasites [194]

1.4.1 Life cycle of *N. brasiliensis*

The life cycle of *N. brasiliensis* in the host begins when infective third stage (L3) larvae penetrate the skin and enter the vasculature. Once in the vasculature, the parasites migrate into the lungs via the circulatory system causing massive damage in the lungs within 24-48 h. Larvae enter the airways, from which they are coughed up and swallowed into the stomach. L3 moult into L4 mature adult worms that produce eggs upon reaching the small intestinal lumen three days after infection. BALB/c mice expel *N. brasiliensis* worms within 14 days post-infection [195]. IL-13 driven Th2 immunity is crucial for conferring protection to *N. brasiliensis* infection [144,145,194]. Secondary infection with *N. brasiliensis* induces a potent memory immune response that results in poor worm maturation and inhibits egg production [196]. The life cycle of *N. brasiliensis* is illustrated in Fig 1.7.

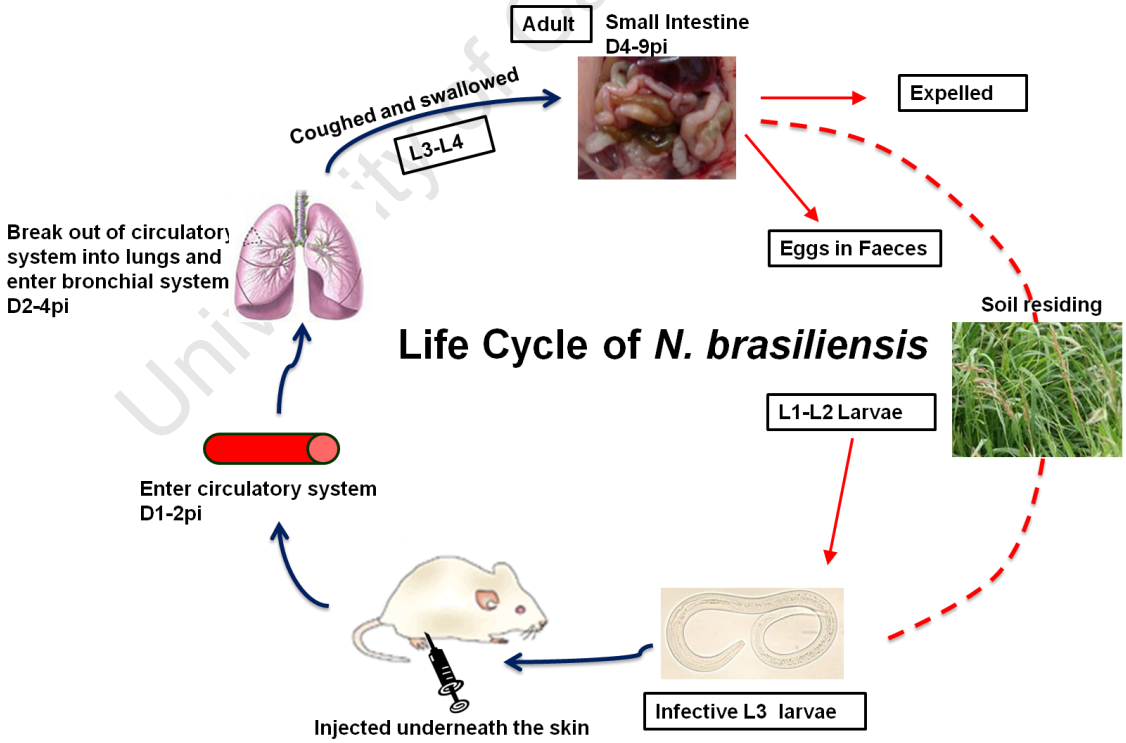

Figure 1.7: Life cycle of *Nippostrongylus brasiliensis*.
Infective third stage *N. brasiliensis* larvae (L3) infect mice and migrate via the circulatory system to the lungs. They enter the airways, get coughed up and swallowed in to the stomach. L3 moults into L4 in the small intestine and begins laying eggs. Immuno-competent mice expel the worms by day 14 post-infection. This figure was adapted from [194]

1.4.2 Immune response to *N. brasiliensis* infection

Protective immunity to *N. brasiliensis* infection is mediated by the development of a dominant Th2 immune response characterised by increased production of Th2 cytokines IL-13 and IL-4 [144,145,197], goblet cell hyperplasia [156,198] eosinophilia [36] and elevated levels of antigen-specific serum IgG1 and IgE [38,145]. Mice deficient of IL-4 were found to expel *N. brasiliensis* worms similarly to immuno-competent wild-type mice, demonstrating that protection occurs independently of IL-4 signalling [199,200]. In contrast, mice deficient of IL-13, IL-4/IL-13, IL-4Rα and STAT-6 failed to resolve *N. brasiliensis* infection, indicating the importance of IL-13 in driving protection [144,145,155,201]. Moreover, in a study from our laboratory, mice lacking IL-4Rα expression specifically on smooth muscle cells failed to expel *N. brasiliensis* worms due to impaired Th2 cytokine responses and reduced goblet cells hyperplasia [189]. Mice lacking alternatively activated macrophages (*LysM*^{cre}IL-4Rα$^{-/lox}$) successfully expelled *N. brasiliensis* adult worms and developed sufficient Th2 responses and goblet cell hyperplasia [147].

1.5 *Schistosoma mansoni*

Schistosomiasis, also known as bilharzia, is a chronic parasitic disease caused by digenetic trematode flatworms (flukes) belonging to the *Schistosoma* genus. The disease infects approximately 200 million people and is endemic in 74 developing countries where it is a measure cause for morbidity and mortality [202,203,204]. The disease is estimated to cause 280 000 deaths per annum in sub-Saharan Africa alone [203], hence its inclusion amongst the top ten infectious diseases of concern by the World Health Organisation (WHO) [202].

Although schistosomiasis can easily be treated with an inexpensive chemotherapeutic drug, praziquantel [205]; the spreading of the disease to new areas is a major concern. Furthermore, treatment of infected individuals does not prevent re-infection, a common occurrence in areas where the disease is endemic. Thus, given the morbidity associated with Schistosomiasis, it is wise to develop a long lasting solution that would protect the hosts from continued infections. This issue has been made more pressing by the emergence of HIV/AIDS, a disease that is highly prevalent in sub-Saharan Africa. There is already evidence suggesting that schistosome infection alters the aetiology and transmission of HIV/AIDS [206,207,208,209,210], tuberculosis [207,211,212] and malaria [213,214,215]. Hence, more work is required to understand the

immunobiology of Schistosomiasis in order to facilitate rational design and development of effective vaccine.

1.5.1 Life cycle

The life cycle of *S. mansoni* begins when infective cercariae penetrate the skin of the human host, transform into schistosomula by losing their tail and then entering the lymphatic vessels and capillaries en route to the lungs. After a few days, the schistosomula migrate into the portal venous system where they mature into adult worms and unite. The worm pairs migrate into the mesenteric venules that drain the intestines of the mammalian host where they lay hundreds of eggs per worm pair per day [216]. Egg production commences 4 to 6 weeks post-infection and last the entire life of the worm pair, which may be up to 15 years in a definitive host [216]. The eggs deposited in the vein lumen pass through the intestinal tissue, enter the gut and are excreted in the faeces. The life cycle is completed when the eggs hatch, releasing miracidia that infect the intermediate host, species-specific freshwater snails (*Biomphalaria* sp. for *S. mansoni* miracidia). After rounds of asexual reproduction within the snail host, infective cercariae are released to begin the life cycle yet again. The life cycle of *S. mansoni* is summarised in Figure 1.8.

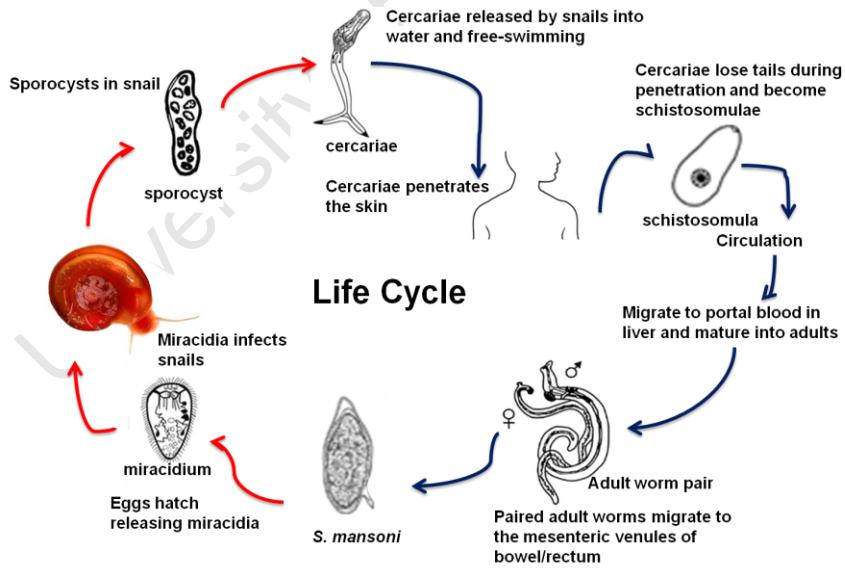

Figure 1.8: Life cycle of *Schistosoma mansoni*.
The life cycle of *S. mansoni* progresses from a definitive mammalian host to an intermediate host, the freshwater snails. The cycle commences when infective cercariae burrow into the skin of the mammalian host, transform into schistosomula, and mature into egg laying adult worm pairs in the mesenteric venules. After mating, female worm produce eggs that traverse the intestinal tissue and finally get excreted in the faeces. Eggs hatch in the water releasing miracidia that infect species-specific freshwater snails. The life cycle was adapted from CDC (www.dpd.cdc.gov/dpdx

1.5.2 Immunobiology of schistosomiasis

The host immune response to *S. mansoni* infection is orchestrated by $CD4^+$ T cells and progresses through three main phases characterised by the type of the dominant immune response (Figure 1.9) [217]. During the first 3-5 weeks of exposure to parasites, a dominant Th1 immune response is triggered by the immature parasites. However, once egg production by the parasites begins at 5-6 weeks post-infection, the Th1 immune response is down-regulated while egg-antigens elicit a strong Th2 response which drives the development of granulomatous lesions around tissue-trapped eggs [217]. The development of a Th2 immune response has been shown to be indispensable for host protection during *S. mansoni* infection [147,218,219]. During the chronic stages of infection (10 weeks onward), the Th2 immune response is down-modulated due to the emergence of IL-10 producing regulatory T cells and granulomas that form around newly deposited eggs are smaller than during the acute infection [220,221,222].

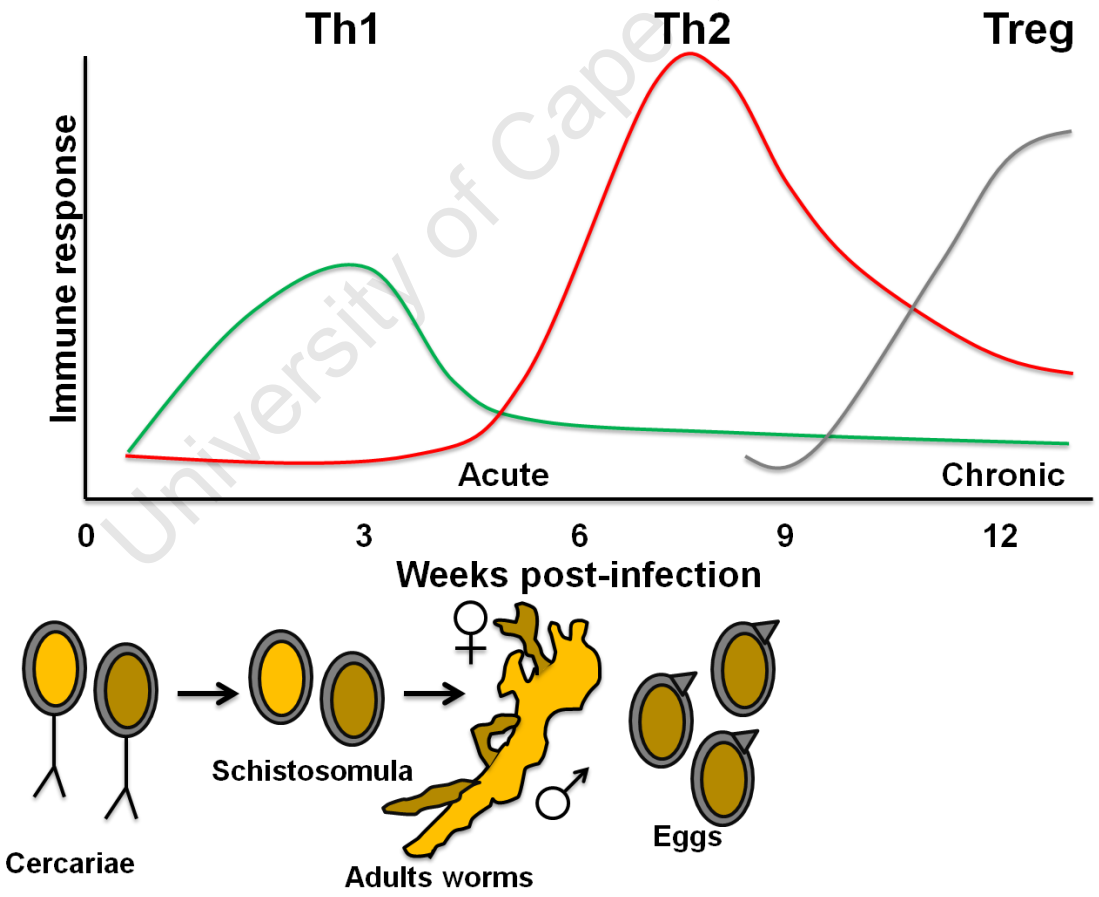

Figure 1.9: The immune response to *S. mansoni* infection.
The immune response to *S. mansoni* infection progressed from a dominant Th1 immune response during the early phases of the infection (3-5 weeks), to a dominant Th2 immune response induced by egg-antigens (5-8 weeks) and finally down-modulation of the Th2 immune response during the chronic stages of infection and emergence of Treg population. The figure was adapted from [217].

1.5.3 Acute vs chronic schistosomiasis

Acute schistosomiasis coincides with egg-laying by adult worms (5-6 weeks post-infection), peaking at 8 weeks post-infection and is characterised by a dominant Th2-polarised cellular immune response induced by *S. mansoni* eggs trapped in the host tissues [217]. Studies have shown that development of a polarised Th2 immune response driven by IL-4 and IL-13 signalling is crucial for granuloma formation, tissue fibrosis and limiting pathological consequences of *S. mansoni* infection [147,148,149,218,219]. However, a study by Hoffmann and colleagues revealed that maintaining a fine balance between Th1 and Th2 immune response is crucial for surviving acute schistosomiasis in mice [222]. A key feature of acute schistosomiasis in susceptible mice is rapid weight loss that coincides with egg-laying and this has been correlated with increased production of proinflammatory cytokines like TNF-α, iNOS and IFN-γ [218,222]. In humans, patients suffering from severe hepatosplenic schistosomiasis exhibit highly elevated levels of IFN-γ, NO and TNF [223].

In mice, chronic schistosomiasis is characterised by development of a Th2-polarised immune response indicated by increased production of profibrotic cytokines IL-4 and IL-13 [224,225,226], reduced production of IFN-γ [227] and significant tissue fibrosis [228]. The persistent granulomatous response and fibrosis eventually leads to portal hypertension, establishment of porto-systemic shunts, intestinal bleeding and ultimately death. In order to circumvent the destructive effects of the host's immune response during the chronic stages of infection, it is crucial to control the immune response triggered by the eggs. Immuno-competent mice are capable of modulating the deleterious effects of the immune response during the chronic stages of *S. mansoni* infection. In these mice, immuno-modulation is indicated by decreased size of newly formed granulomas, reduced fibrosis and controlled T cell responsiveness to egg antigens. The mechanisms mediating down-modulation of granulomatous hypersensitivity in schistosomiasis have been linked to $CD8^+$ suppressor cells [229] and cross-regulatory cytokines produced by $CD4^+$ T cells [229,230,231]. However, subsequent studies demonstrated a pivotal role played by B cells in down-regulating granuloma formation during chronic Schistosomiasis [232,233]. Although there is a lot of contradictory data, IL-10 produced by B cells has been proposed as a key immuno-modulatory cytokines during chronic Schistosomiasis [231,234]. IL-10 has been implicated in limiting pathological events associated with severe chronic disease [220,222,235]. In a study by Sadler and colleagues, it was shown that IL-$10^{-/-}$ mice suffer from severe inflammation during the chronic stage of infection and fail to down-modulate the cellular immune

responses. A recent study by Fairfax and colleagues showed that impairing IL-10R signalling results in severe pulmonary disease and loss of B cells in the liver during chronic schistosomiasis [221].

In humans, the life-threatening form of chronic schistosomiasis occurs at a frequency of 5-10% in untreated populations and is characterised by hepatosplenic disease accompanied by severe hepatic and periportal fibrosis, pulmonary hypertension and portosystemic shunting of venous blood [217,223,236,237]. Patients with severe disease were found to have Th1-like responses and high levels of plasma TNF receptor I (TNFRI) and TNFRII, whereas patients with less severe disease developed Th2-like responses and down-regulated plasma levels of TNF receptors [238]. The severity of disease has been shown to be dependent on the intensity of infection and most importantly, genetic predisposition of the individuals [239,240]. A study conducted by Dessein and colleagues in *S. mansoni* endemic areas in Sudan found that certain families were more likely to develop severe hepatic fibrosis and portal hypertension than others and this was linked to a codominant major gene known as *SM2* [239]. *SM2* encodes for IFN-γ receptor 1 (IFN-γR1) and data suggests that polymorphisms in IFN-γR1 leads to augmented fibrosis due to the inability of IFN-γ to inhibit fibrogenesis because the receptor has lost its function [217,239].

1.5.4 Mechanism conferring protection against *S. mansoni* infection

Earlier studies using gene-deficient mice have been instrumental in elucidating factors contributing to host protection or susceptibility during *S. mansoni* infection in mice. Mice deficient of IL-4 (IL-$4^{-/-}$) were found to be extremely susceptible to *S. mansoni* infection and death was shown to be preceded by rapid cachexia coincident with egg-laying by adult worms [218]. Furthermore, IL-4/IL-10 double deficient mice were found to be more susceptible to *S. mansoni* infection than IL-$4^{-/-}$ mice due to augmented production of pro-inflammatory cytokines IFN-γ and TNF-α, increased hepatocellular damage indicated by elevated levels of aspartate transaminase (AST), nitric oxide (NO) production and destruction of gut integrity [222]. On the contrary, IL-10/IL-12 deficient mice did not suffer from severe weight loss during the acute stage of infection but displayed a progressive wasting disease that culminated in death during the chronic stages of *S. mansoni* infection [222]. This study demonstrated that excessive Th1 or Th2 cytokine responses trigger distinct, but equally detrimental forms of disease following infection [222]. Hence, maintaining a fine balance between Th1 and Th2 immune response is crucial for surviving

schistosomiasis in mice. Furthermore, IL-10 has emerged a key immunoregulatory factor controlling pathogenesis during *S. mansoni* infection [222].

IL-4Rα$^{-/-}$ and STAT-6$^{-/-}$ mice are highly susceptible to *S. mansoni* infection due to impaired granuloma formation, diminished Th2 cytokine production, reduced fibrosis and exacerbated tissue inflammation [148,149]. The importance of cell-specific IL-4Rα expression in the mechanism conferring protection or susceptibility to *S. mansoni* infection has been investigated in our laboratory by utilising cell-specific IL-4Rα-deficient mice generated using the Cre/*lox*P recombination system. Mice deficient in IL-4Rα expression on macrophages and neutrophils (*LysM*^{cr}eIL-4Rα$^{-/lox}$) were found to be highly susceptible to acute Schistosomiasis despite the presence of the Th2 immune response [147]. Mortality in *LysM*^{cr}eIL-4Rα$^{-/lox}$ mice was caused by uncontrolled liver damage and excessive gut inflammation which ultimately led to endotoxemia and septic shock [147]. Mice lacking IL-4Rα expression on pan-T cells (*iLck*^{cre}IL-4Rα$^{-/lox}$) [190] and smooth muscle cells (SMC^{cre}IL-4Rα$^{-/lox}$) [188] were found to be susceptible to *S. mansoni* infection despite controlling gut inflammation. The contribution of IL-4/IL-13 responsive B cells and CD11c^{+} dendritic cells in the mechanism conferring host protection or susceptibility to acute schistosomiasis are yet to be determined.

1.6 Objectives of the present study

1. To investigate the role of CD28 costimulation during recall of protective immunity against *N. brasiliensis* secondary infection.
2. To determine the contribution of IL-4/IL-13 responsive B cells in host protection or susceptibility during acute and chronic schistosomiasis.
3. To investigate the role of IL-4/IL-13 responsive CD11c^{+} dendritic cells during infection with *S. mansoni*.

Chapter 2

CHAPTER 2

CD28 is required for protection against *Nippostrongylus brasiliensis* secondary infection and recall of memory responses in inducible CD28 deleting mice.

H.H. Ndlovu[1#], M. Darby[1], M. Froelich[2], W. Horsnell[1], F. Lühder[3] T. Hünig[2] and F. Brombacher[1*]

[1] International Center for Genetic Engineering and Biotechnology (ICGEB), Cape Town Component and Institute of Infectious Diseases and Molecular Medicine (IIDMM), Division of Immunology, University of Cape Town, Cape Town, South Africa ,
[2] Institute for Virology and Immunology, University of Würzburg, Würzburg, Germany,
[3] Department of Neuroimmunology, Institute of Multiple Sclerosis Research and The Hertie Foundation, University Medical Center Göttingen, Germany.

ABSTRACT

IL-13 driven Th2 immunity is indispensable for host protection against infection with the gastrointestinal nematode *Nippostronglus brasiliensis*. Disruption of CD28 mediated costimulation impairs development of adequate Th2 immunity, showing an importance for CD28 during the initiation of an immune response against this pathogen. However, any requirement for CD28 during recall immunity to secondary infection with pathogens has remained largely controversial. In this study, we used global $CD28^{-/-}$ mice and a novel mouse model that allows for inducible deletion of the *cd28* gene by oral administration of tamoxifen ($CD28^{-/lox}Cre^{+/-}$+TM) to address these questions. Following primary infection with *N. brasiliensis*, $CD28^{-/-}$ mice failed to expel adult worms in the small intestine in contrast to wild-type C57BL/6 mice. Impaired expulsion was associated with reduced production of Th2 cytokines, particularly IL-13, reduced serum levels of antigen specific IgG1 and total IgE and failure to develop $CXCR5^{+}$ T_{FH} cells. Interestingly, interference with CD28 expression in $CD28^{-/lox}Cre^{+/-}$ mice by oral administration of tamoxifen prior to secondary infection with *N. brasiliensis* resulted in impaired worm expulsion. This correlated with reduced production of Th2 cytokines IL-13 and IL-4, diminished serum titres of antigen specific IgG1 and total IgE and reduced $CXCR5^{+}$ T_{FH} cell population. Importantly, abrogation of CD28 during secondary infection inhibited the recruitment of central and effector memory $CD4^{+}$ T cells and follicular B cells to the draining lymph node. Therefore, it can be concluded that CD28 is essential for the development of protective memory Th2 immunity against *N. brasiliensis* infection and for expansion of $CXCR5^{+}$ T_{FH} cells and follicular B cells.

Keywords: CD28, tamoxifen, recall, *N. brasiliensis*, mice.

2.1 INTRODUCTION

Infection of mice with *Nippostrongylus brasiliensis* triggers a host protective immune response characterised by increased production of Th2 cytokines IL-13 and IL-4 [144,145,197], goblet cell hyperplasia [198] eosinophilia [36] and elevated levels of serum IgG1 and IgE [38,145]. Infection with *N. brasiliensis* begins when L3 larvae penetrate the skin and migrate, via the circulatory system, into the lungs. Larvae enter the airways, from which they are coughed up and swallowed. The larvae mature into adult worms that produce eggs upon reaching the small intestinal lumen. Immune-competent BALB/c mice clear *N. brasiliensis* infection after approximately 9 days post-infection [195]. Secondary infection with *N. brasiliensis* induces a potent memory immune response that results in poor worm maturation and inhibits egg production [196].

CD28 is considered to be the main co-stimulator of T cells, providing a critical signal for activation of naive T cells [51,52,241]. Interactions between CD28 and its ligands CD80/CD86 enhances cytokine production, prevents T cell anergy and protects against apoptosis [53,242]. These CD28 dependent interactions are important during the initiation of T cell mediated immunity against a number of infections. Mice deficient in CD28 failed to develop adequate Th2 immune response during infection with *S. mansoni* [57], *L. major* [58] and *N. brasiliensis* [66,67]. In contrast, infection of $CD28^{-/-}$ mice with *H. polygyrus* did not hamper normal development of Th2 immune response [243].

The absence of CD28 alters the organisation of secondary lymphoid tissue by affecting recruitment of T cells to B cell follicles, impairing germinal centre development [54,55,56], isotype switching, B cell maturation and development of memory B cells. This is linked to diminished recruitment of $CXCR5^{+}$ T_{FH} cells which localise within the B cell follicles [59,60,61,62]. T_{FH} cells produce IL-21, a key cytokine involved in isotype switching and differentiation of plasma cells [60]. $CD28^{-/-}$ mice infected with *S. mansoni* [57] or *Leishmania major* [58] failed to produce antigen specific type 2 antibodies IgG1 and IgE. Taken together this demonstrates an important role for CD28 in co-ordinating B cell responses.

Studies suggest that CD28 is not required during recall of memory T cell responses to infection with *N. brasiliensis* [66] and *H. polygyrus* [69]. Furthermore, infection of $CD28^{-/-}$ mice with fungi *B. dermatitidis* revealed maintenance of memory T cells is CD28 independent [68]. In fact, some studies suggested that recall memory responses may be dependent on other co-stimulatory molecules such as inducible costimulator (ICOS) or 4-1BB [244,245,246]. Contrastingly, development of effector and memory $CD4^{+}$ T cells was reduced in the absence of CD28 during *T. gondii* infection [70]. Recall of memory responses to persistent viral infections is dependent on CD28 [71,72]. Therefore, the importance of CD28 during development and recall of memory responses remains controversial. There have been attempts to address this issue by blocking CD80 and CD86 or by transfer of memory T cells into CD80/CD86 deficient mice [247]. However, both

approaches deprive CTLA-4 (CD152) of its ligands thus caution must be exercised when interpreting these data. Hence, new approaches that don't suffer from these additional effects are required to solve the conundrum surrounding the contribution of CD28 during recall of memory responses to infections.

The aim of this study was to evaluate the importance of CD28 in initiating protective Th2 immunity against both primary and secondary infections with *N. brasiliensis*. Our findings demonstrate that CD28 is required for initiation of protective Th2 immunity against primary infection with *N. brasiliensis*. Furthermore, the absence of CD28 impairs development of memory $CD4^+$ T cell responses resulting in failure to clear adult *N. brasiliensis* worms during secondary infection. Failure to resolve infection was associated with reduced production of Th2 cytokines particularly IL-13 and IL-4, abrogated humoral immunity and failure to expand $CXCR5^+$ T_{FH} cells.

2.2 Materials and Methods

Mice. C57BL/6 background $CD28^{-/-}$ and $CD28^{-/lox}Cre^{+/-}$ were obtained from Prof. T. Hünig at the University of Würzburg, Germany. The mice were bred and maintained in specific pathogen-free barrier conditions in individually ventilated cages at the University of Cape Town Animal Facility. All experimental mice were age and sex matched and used between 8-12 weeks of age.

Ethics statement. This study was conducted under strict recommendation of the South African national guidelines and of the University of Cape Town practice for laboratory animal procedures. All mouse experiments were carried out in accordance to protocols approved by the Animals Research Ethics Committee of the Health Sciences Faculty, University of Cape Town (Project Number: 008/011). Care was taken to minimize suffering of the animals.

N. brasiliensis infection

Primary Infection. Mice were injected subcutaneously with 500 *N. brasiliensis* L3 larvae. Mice were killed nine days post-infection and adults worms were enumerated using a previously described method [144].

Secondary Infection. Mice were initially injected with 500 *N. brasiliensis* L3 larvae, orally treated with 10 mg/ml Ivermectin in drinking water at seven days post-infection and shelved for 21 days prior to a secondary subcutaneous infection with 500 *N. brasiliensis* L3 larvae. Mice were killed 5 days post secondary infection by halothane inhalation and exsanguination.

Histology. Tissue sample were fixed in buffered 4% (v/v) formaldehyde, embedded in paraffin wax and cut into 5µm sections. The sections were stained with periodic acid-Schiff reagent (PAS)

in order to visualize mucus producing goblet cells. The sections were analysed under a light microscope.

Determination of antibody titres. *N. brasiliensis* antigen-specific serum antibody isotypes and total IgE titres from infected mice were determined as previously described [248]. Briefly, blood was collected in serum separator tubes (BD Bioscience, San Diego, CA) and centrifuged at 8 000×g for 10 min at 4ºC to separate serum. The flat-bottom 96-well plates were coated with 10 µg/ml somatic *N. brasiliensis* antigen (NAg), blocked with 2% (w/v) milk powder for 2 h at 37ºC and samples were loaded and incubated overnight at 4ºC. Alkaline phosphatase labelled secondary antibody was added and incubated for 2 h at 37ºC. The plates were developed by addition of 4-nitrophenyl substrate (Sigma). The absorbance was read at 405nm using VersaMax microplate spectrophotometer (Molecular Devices, Germany).

***Ex vivo* restimulation of cells and cytokines detection.** Single cell suspensions were prepared by pressing the draining lymph nodes through 70 µM cell-strainers. Cells were resuspended in complete IMDM (Gibco) supplemented with 10% FCS (Gibco) and penicillin and streptomycin (100 U/ml and 100 µg/ml, Gibco). The cells were cultured at 2×10^6 cells/ml in 48-well plates coated with α-CD3 (20 µg/ml) or NAg (20 µg/ml) plus sub-optimal concentration of α-CD3 (2 µg/ml) and incubated at 37ºC in a humidified atmosphere containing 5% CO_2. Supernatants were collected after 72 h and cytokines were measured by ELISA. Quantities of IL-4, IL-10 and IL-13 were measured by sandwich ELISA as previously described [248].

Inducible deletion in conditional knock-out mice. $CD28^{-/lox}Cre^{+/-}$ mice were given 2.5 mg Tamoxifen (Sigma, Deisenhofen, Germany) in vegetable oil for four consecutive days by forced feeding.

Flow cytometry. The following antibodies comprising the B cells antibody panel were used: B220-V500, CD19-PerCP Cy5.5, CD23-PE, CD21-APC, CD24-PE Cy7, CD80-V450, MHCII-FITC and IgM-Biotin (BD Bioscience, Erembodegem, Belgium). T cells panel consisted of the following antibodies: CD4-PerCP, CD3-AlexaFluor 700, CD62L-V500, CD44-FITC, CD28-PE, CXCR5-V450 and CD278-Biotin (BD Bioscience, Erembodegem, Belgium). Cells were acquired on a FACS Fortessa machine (BD Immunocytometry system, San Jose, CA, USA) and data was analyzed using Flowjo software (Treestar, Ashland, OR, USA).

Statistics: Statistical analysis was conducted using GraphPad Prism 4 software (http://www.prism-software.com). Data were calculated as mean ± SD. Statistical significant was determined using the unpaired Student's *t* test or 2-way ANOVA with Bonferroni's post test,

defining differences to C57BL/6 or $CD28^{-/lox}Cre^{+/-}$ given oil as significant (*,$p \leq 0.05$; **,$p \leq 0.01$; ***,$p \leq 0.001$)

2.3 RESULTS

CD28 costimulatory signalling is required for protective immunity against *N. brasiliensis* infection.

In order to investigate whether CD28 is required for development of protective immunity against *N. brasiliensis* infection, $CD28^{-/-}$ mice were infected with 500 L3 *N. brasiliensis* worms and killed 9 days post-infection (Fig 2.1A). $CD28^{-/-}$ mice had significantly higher intestinal adult worm burdens compared to wild-type C57BL/6 mice (Fig 2.1.B). CD28 deficiency did not affect T_H2 associated goblet cells hyperplasia (Fig 2.1C), a possible mechanism mediating protective immunity against parasitic nematode infections [189]. Protective immunity to *N. brasiliensis* infection is dependent on the production of Th2 cytokines, particularly IL-13 [145]. Analysis of cytokine production after re-stimulation of total mesenteric lymph node (MLN) cells with *Nippostrongylus* antigen (Nag) in the presence of sub-optimal concentration of α-CD3 showed significantly reduced production of IL-13 and IL-10 (Fig 2.1D) in infected $CD28^{-/-}$ mice when compared to infected C57BL/6 mice. Furthermore, restimulation of MLN cells with α-CD3 only, resulted in diminished production of the key cytokines IL-13 and IL-4 in $CD28^{-/-}$ mice compared with C57BL/6 mice (Fig 2.1E). However, production of IFN-γ was not affected by the deficiency of CD28 costimulatory signalling on MLN cells (Fig 2.1E). $CD28^{-/-}$ mice up-regulated the expression of IL-6 after restimulation with either NAg plus sub-optimal concentration of α-CD3 or CD3 alone compared to wild-type control mice. Finally, production of *N. brasiliensis* specific IgG1 was not altered whereas total IgE was markedly reduced in mice lacking CD28 signalling on T cells (Fig 2.1F & G). The production of *N. brasiliensis* specific type 1 antibodies (IgG2a and IgG2b) was also reduced in $CD28^{-/-}$ mice compared with C57BL/6 mice (Figure 2.2), suggesting that CD28 deficiency results in a general attenuation of the humoral immune response during nematode infection. Together, these data reveal that CD28 is required for protective immunity against *N. brasiliensis* primary infection.

CD28 costimulation is crucial for development of T_{FH} cells and effector $CD4^+$ T cells during primary infection with *N. brasiliensis*.

To investigate the impact of CD28 deletion on the differentiation of $CD4^+$ T cells, we analysed different $CD4^+$ T cells subsets by flow cytometry after primary *N. brasiliensis* infection. Surface expression of CD28 by $CD3^+CD4^+$ T cells from $CD28^{-/-}$ mice was impaired compared to C57BL/6 mice (Fig 2.3A). There was no significant difference in the total number of $CD3^+CD4^+$ T cells between C57BL/6 and $CD28^{-/-}$ mice (Fig 2.3B). However, the absolute numbers of follicular helper T cells (T_{FH}) defined by the expression of CXCR5 (Fig 2.3C) and ICOS (Fig 2.3D) were significantly reduced in $CD28^{-/-}$ mice compared to wild-type mice. There was similar numbers of naive $CD4^+$ T cells ($CD4^+CD44^-CD62L^+$) and central memory $CD4^+$ T cells

($CD4^+CD44^+CD62L^+$) between the mutant and control mice (Fig 2.3E & F). Interestingly, the absolute numbers of effector $CD4^+$ T cells was significantly reduced in $CD28^{-/-}$ mice compared to C57BL/6 mice (Fig 2.3G). Together, these data suggest a critical requirement for CD28 costimulation for the expansion of T_{FH} cells and effector $CD4^+$ T cells.

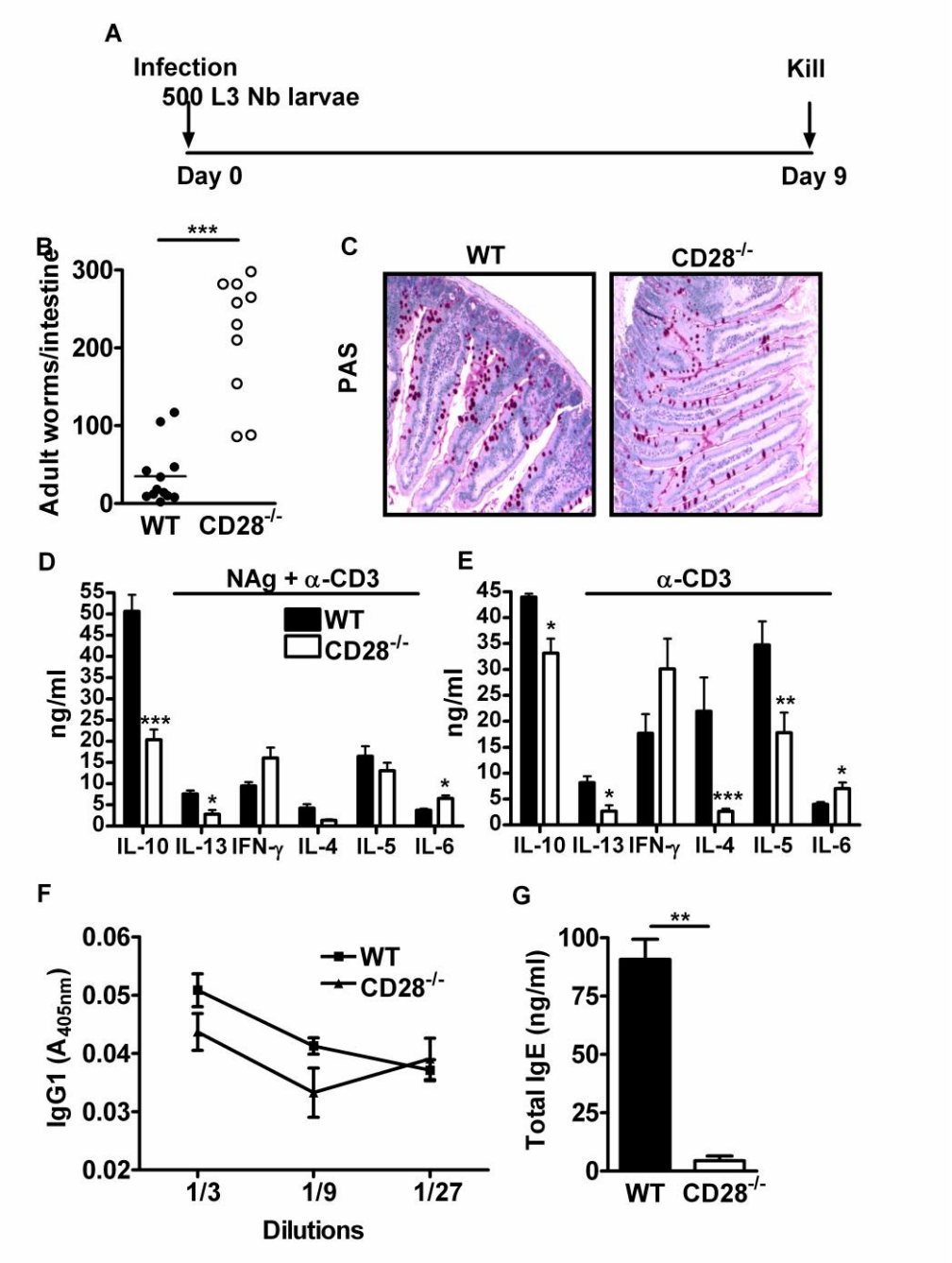

Figure 2.1: CD28 is required for the development of protective immunity against *N. brasiliensis* primary infection.

Wild-type (C57BL/6) and $CD28^{-/-}$ mice were infected with 500 L3 *N. brasiliensis* larvae and killed 9 days post-infection. (**A**), Schematic showing experimental set-up. (**B**), Intestinal worm burdens were quantified. (**C**), PAS staining of mucus producing goblet cells in the intestinal tissue. (**D-E**), Cytokine production by total mesenteric lymph node cells re-stimulated with 20 µg/ml NAg plus sub-optimal concentration of α-CD3 (2 µg/ml) or 20 µg/ml α-CD3 only was determined by ELISA. (**F-G**), Serum antibody titres of *N. brasiliensis* specific IgG1 and total IgE were determined by ELISA. Data are representative of two independent experiments. n= 4-6 mice per group. **P*<0.05, ***P*<0.001, and ****P*<0.0001 vs C75BL/6 mice.

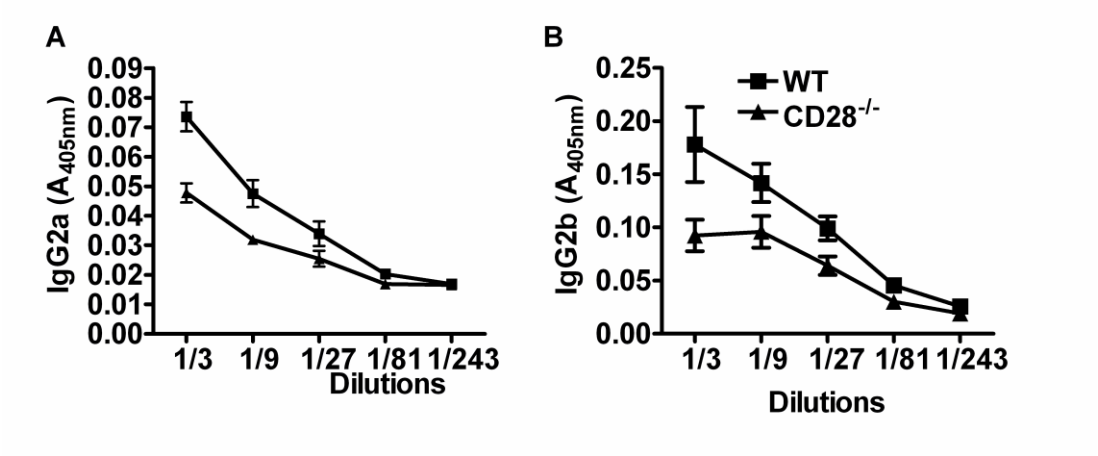

Figure 2.2: Diminished serum titres of type1 antibodies in the absence of CD28 during infection with *N. brasiliensis*.
Wild-type (C57BL/6) and CD28$^{-/-}$ mice were infected with 500 L3 *N. brasiliensis* larvae and killed 9 days post-infection. Blood was collected, spun at 8 000×g for 10 min to separate the serum. (**A-B**), Serum antibody titre of *N. brasiliensis* specific IgG2a and IgG2b was determined by ELISA. Data are representative of two independent experiments. n= 4-6 mice per group.

CD28 is required for protective memory responses to *N. brasiliensis* infection.

CD28 is indispensable for optimal development of Th2 immune responses, however, it significance in disease protection is unclear. Therefore, to investigate the contribution of CD28 in host protective memory responses, we took advantage of a novel inducible CD28 deleting mouse strain (CD28$^{-/lox}$Cre$^{+/-}$), a suitable experimental model system, recently developed by us [249,250]. CD28$^{-/lox}$ mice (exon 2 and 3 are flanked by *lox*P) were intercrossed with rosaCreER^{T2} mice to generate CD28$^{-/lox}$rosaCreER^{T2} mice. Oral administration of estrogen analogue tamoxifen allowed for translocation of estrogen-receptor-Cre fusion protein into the nucleus, where Cre carried out efficient deletion of *cd28* gene [249,250,251].

CD28$^{-/lox}$Cre$^{+/-}$ and CD28$^{-/-}$ mice were infected with 500 L3 *N. brasiliensis* larvae, treated with Ivermectin and rested for 21 days (Fig 2.4A). At day 29, CD28$^{-/lox}$Cre$^{+/-}$ mice were either treated with tamoxifen in vegetable oil (CD28$^{-/lox}$Cre$^{+/-}$+TM) or oil only (CD28$^{-/lox}$Cre$^{+/-}$, control) for four consecutive days (Fig 2.4A). At day 35, mice were re-infected with 500 L3 *N. brasiliensis* larvae and killed 5 days post-infection (Fig 2.4A). Efficient deletion of CD28 was confirmed by flow cytometric analysis of CD3^{+}CD4^{+} T cells. Here, oral administration of tamoxifen in CD28$^{-/lox}$Cre$^{+/-}$ mice, resulted in similar levels of CD28 expression as was found in infected global CD28$^{-/-}$ mice (Fig 2.5A). CD28$^{-/lox}$Cre$^{+/-}$ mice treated with tamoxifen before challenge with *N. brasiliensis* adult worms exhibited high intestinal worm burdens equivalent to CD28$^{-/-}$ mice (Fig 2.4B). Both tamoxifen treated CD28$^{-/lox}$Cre$^{+/-}$ and CD28$^{-/-}$ mouse groups had significantly higher worm burdens than sham-treated CD28$^{-/lox}$Cre$^{+/-}$ control mice (Fig. 2.4B). Goblet cell hyperplasia was similar in all strains (Fig 2.4B). *In vitro* restimulation of total MLN cells with NAg in the presence of sub-optimal concentration of α-CD3 or optimal concentration of α-CD3 showed markedly reduced production of Th2 cytokines IL-13 and IL-4 in infected CD28$^{-/-}$ and tamoxifen-

treated $CD28^{-/lox}Cre^{+/-}$ mice (Fig 2.4D and E). Serum antibody levels for type 2 antibodies were also significantly reduced in mutant mouse strains compared with sham-treated $CD28^{-/lox}Cre^{+/-}$ control mice (Fig 2.4F and G). Together, these results suggest that memory and subsequent production of protective Th2 cytokines and humoral immune responses are dependent on CD28 during the secondary infection with *N. brasiliensis*.

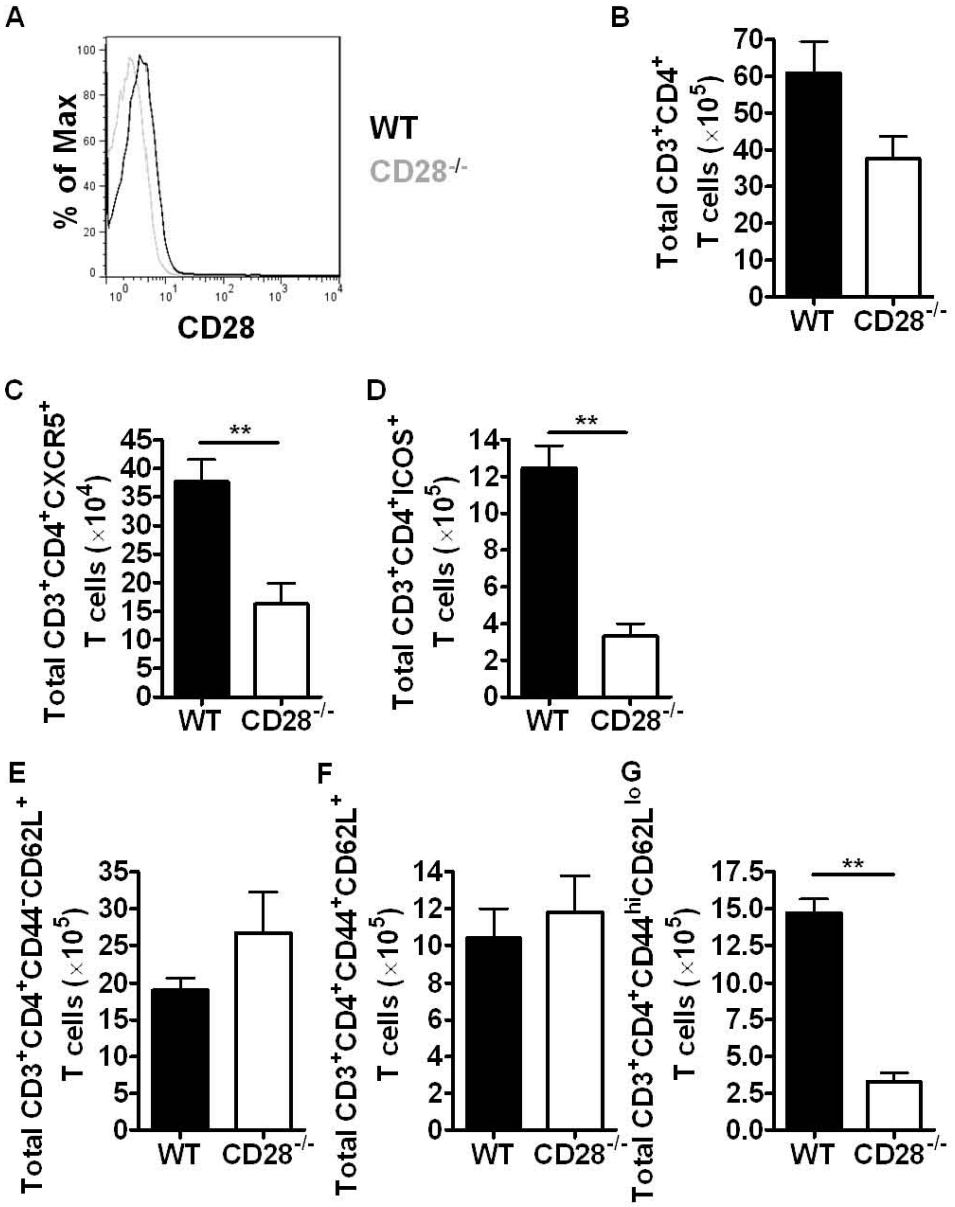

Figure 2.3: Differentiation of T_{FH} cells and effector $CD4^+$ T cells is dependent on CD28 costimulation.
Single cell suspension was prepared from mesenteric lymph node and cells were stained for FACS. (**A**), Histogram showing efficiency of CD28 deletion on $CD3^+CD4^+$ T cells. (**B**), Absolute numbers of $CD3^+CD4^+$ T cells in the lymph node. Absolute numbers of $CD3^+CD4^+CXCR5^+$ (**C**) and $CD3^+CD4^+ICOS^+$ (**D**) T cells recruited to the MLN. (**E-G**) Absolute numbers of T cell subsets infiltrating the draining lymph node. T cell subsets were differentiated based on the following markers: naive ($CD3^+CD4^+CD44^-CD62L^+$), effector ($CD3^+CD4^+CD44^{hi}CD62L^{lo}$) and central memory ($CD3^+CD4^+CD44^+CD62L^+$) T cells. Data represents three independent experiments. n= 4-6 mice per experiment. $^{**}P<0.001$ vs C57BL/6 mice.

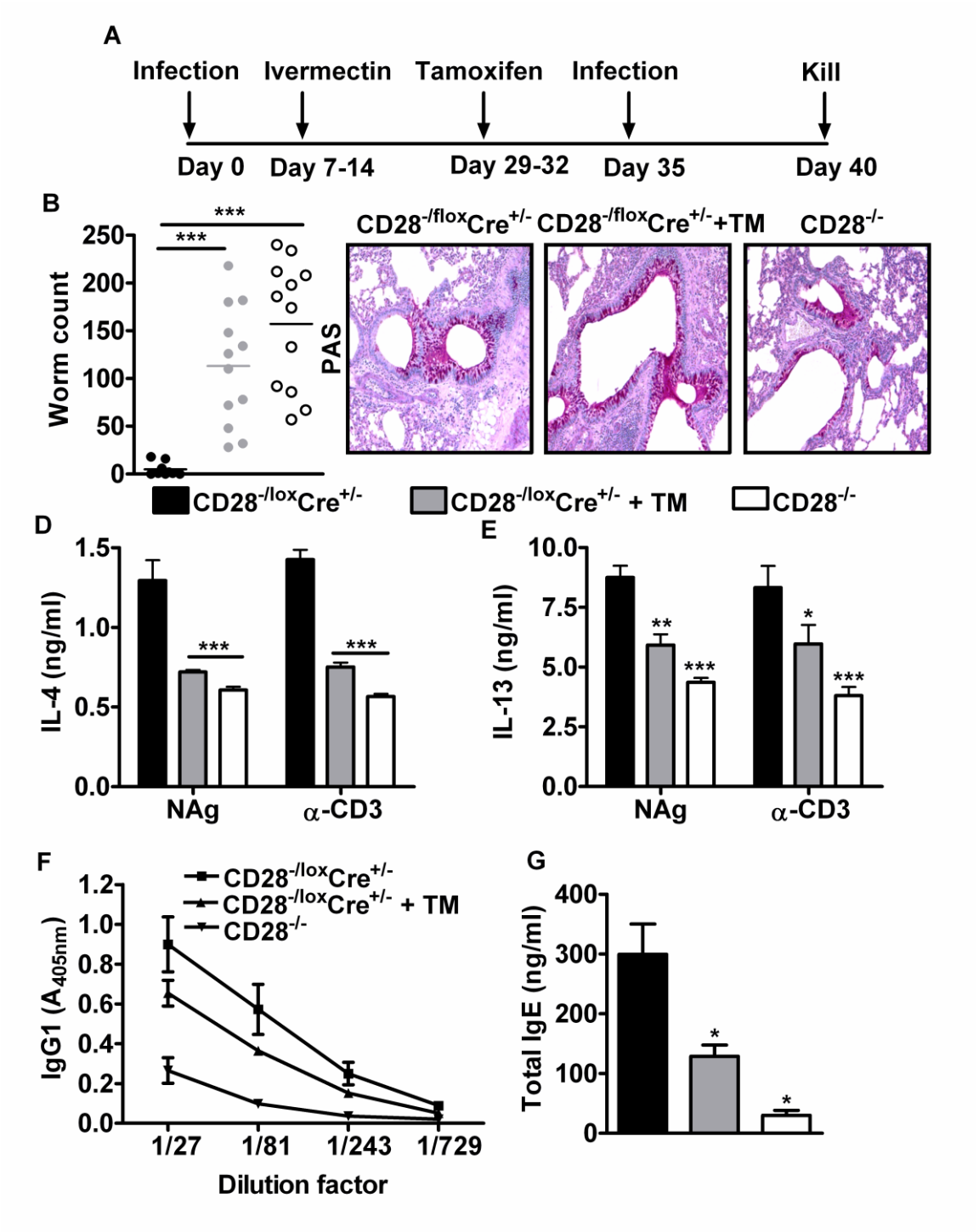

Figure 2.4: CD28 is required for development of protective recall responses to re-infection with *N. brasiliensis*. Wild-type ($CD28^{-/lox}Cre^{+/-}$), tamoxifen treated $CD28^{-/lox}Cre^{+/-}$ and $CD28^{-/-}$ mice were secondary infected with 500 L3 *N. brasiliensis* and killed 5 days post-infection. (**A**), Schematic showing experimental set-up. (**B**), Intestinal worm burdens were quantified. (**C**), PAS staining of pulmonary mucus producing goblet cells (**D-E**), Cytokine production by total mediastinal lymph node cell re-stimulated with either 20 µg/ml NAg plus sub-optimal concentration of α-CD3 (2 µg/ml) or optimal concentration of α-CD3 (20 µg/ml) was determined by ELISA. (**F-G**) Serum antibody titres of *N. brasiliensis* specific IgG1 and total IgE were determined by ELISA. Data is representative of three independent experiments. n= 4-6 mice per group. .*P<0.05, **P<0.001, and ***P<0.0001 vs $CD28^{-/lox}Cre^{+/-}$ mice given oil.

Development of T_{FH} cells, memory and effector $CD4^+$ T cells requires CD28 during secondary infection with *N. brasiliensis*.

To further investigate possible cellular mechanisms, $CD4^+$ T cell subsets were compared at 5 days post secondary infection. The absolute numbers of $CD4^+$ T cells was significantly reduced in tamoxifen-treated $CD28^{-/lox}Cre^{+/-}$ mice compared to control mice, while there was no difference in the number of $CD4^+$ T cells between global $CD28^{-/-}$ mice and control mice (Fig 2.5B). Similar as in global $CD28^{-/-}$ mice, absolute numbers of $CXCR5^+$ and $ICOS^+$ T_{FH} cells were significantly reduced in $CD28^{-/lox}Cre^{+/-}$ mice given tamoxifen compared to sham-treated $CD28^{-/lox}Cre^{+/-}$ mice (Fig 2.5C and D). These data demonstrate that the development of T_{FH} cells during recall responses to *N. brasiliensis* infection is critically reliant to CD28 costimulation during secondary infection only.

There is evidence suggesting that memory T cells develop directly from effector $CD4^+$ T cells [252]. Flow cytometric analysis of memory T cells was conducted to investigate the development of memory T cells during recall responses to nematode infections. The total numbers of naive $CD4^+$ T cells ($CD4^+CD44^-CD62L^+$) were similar in both $CD28^{-/-}$ mice and $CD28^{-/lox}Cre^{+/-}$ mice given tamoxifen during recall responses to *N. brasiliensis* infection (Fig 2.5E). However, the absolute numbers of effector T cells ($CD4^+CD44^{hi}CD62L^{lo}$) were significantly reduced in both $CD28^{-/lox}Cre^{+/-}$ mice given tamoxifen and $CD28^{-/-}$ mice compared with $CD28^{-/lox}Cre^{+/-}$ mice given oil (Fig 2.5F). Moreover, the absolute numbers of central memory T cells ($CD4^+CD44^+CD62L^+$) were also significantly reduced in both mutant mouse strains, when compared with control mice (Fig 2.5G). Together, these data suggest that CD28 is essential for the development of both effector $CD4^+$ T cells and a new pool of central memory $CD4^+$ T cells during re-infection with *N. brasiliensis*.

CD28 influences B cell development in the draining lymph node during secondary infection with *N. brasiliensis*.

Impairment of CD28 T cell costimulation also influenced humoral immunity, hence different subsets of B cells were analysed by flow cytometry in the mediastinal lymph node after secondary infection with *N. brasiliensis*. Global $CD28^{-/-}$ mice showed normal total $CD19^+B220^+$ B cells (Fig 2.6A), strikingly reduced follicular B cells (Fig. 2.6B), more than 6-fold increased marginal zone B cells (Fig 2.6C) and normal numbers of non-follicular B cells (Fig 2.6D) compared to sham-control $CD28^{-/lox}Cre^{+/-}$ mice. This suggests that development of follicular and marginal zone B cells may be dependent on CD28 costimulation. $CD28^{-/lox}Cre^{+/-}$ mice given tamoxifen during secondary infection, presented reduced number of $CD19^+B220^+$ B cells (Fig. 2.6A) due to strikingly reduced follicular and non-follicular B cells (Fig. 2.6B, D), compared to sham-control $CD28^{-/lox}Cre^{+/-}$ mice. The absolute numbers of marginal zone B cells was slightly but significantly increased in $CD28^{-/lox}Cre^{+/-}$ mice treated with tamoxifen compared to sham-treated control mice (Fig 2.6C). The similarly reduced numbers of follicular B cells in $CD28^{-/-}$ mice and $CD28^{-/lox}Cre^{+/-}$ mice given tamoxifen during secondary infection, suggests that development of follicular B cells

is particularly dependent on CD28 costimulation during secondary infection. Moreover, the results further suggest that impairment of CD28 costimulation during primary and/or secondary infection influences follicular and non-follicular B cells. Together, we concluded that CD28 costimulation is important for B cell development in the draining lymph node during re-infection with *N. brasiliensis*.

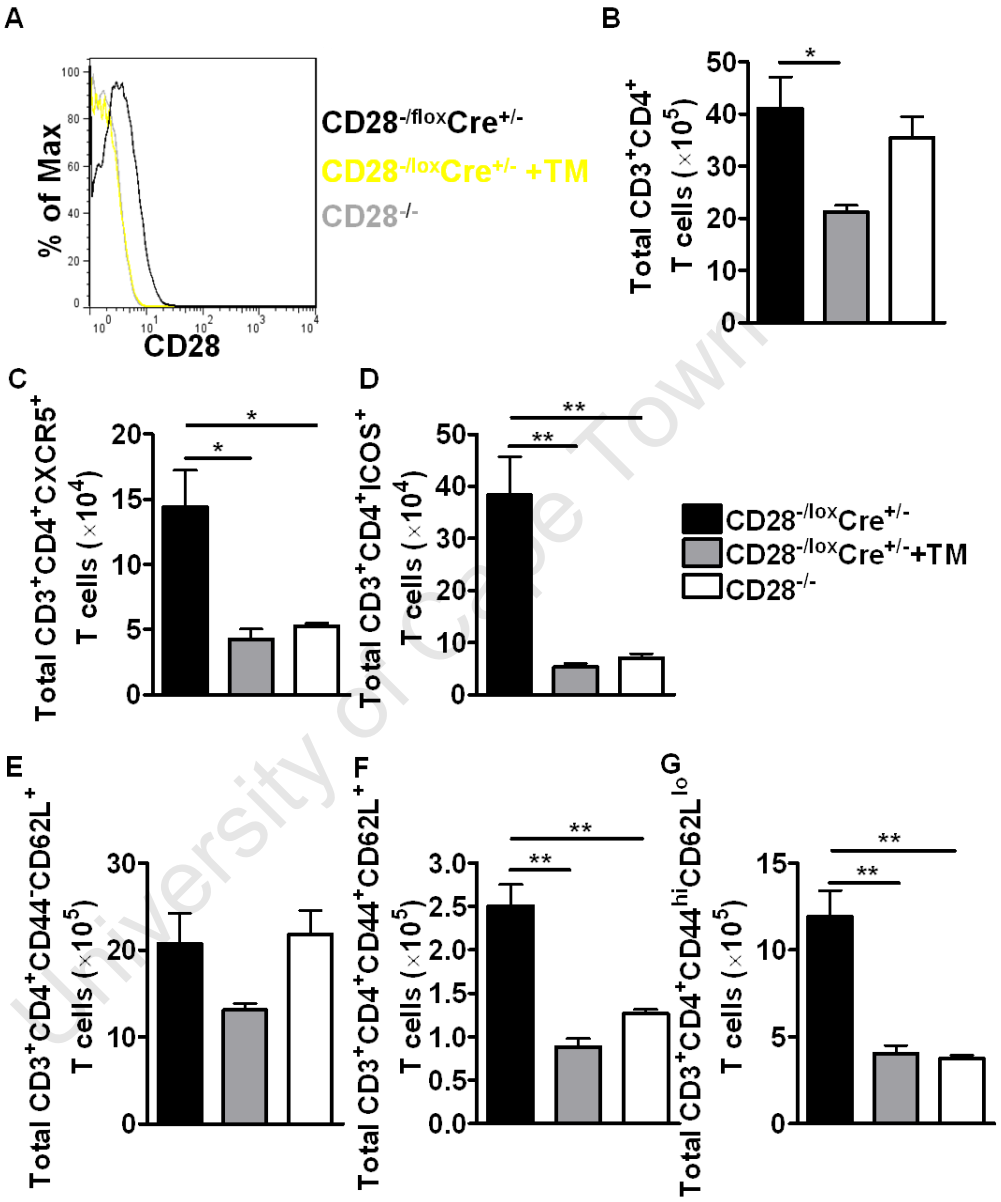

Figure 2.5: CD28 is necessary for the development of T_{FH} cells and optimal activation of $CD4^+$ T cells. Single cell suspension was prepared from mediastinal lymph node and cells were stained for FACS. (**A**), Histogram showing efficiency of CD28 deletion on $CD3^+CD4^+$ T cells. (**B**), Absolute numbers of $CD3^+CD4^+$ T cells in the lymph node. Numbers of $CD3^+CD4^+CXCR5^+$ (**C**) and $CD3^+CD4^+ICOS^+$ (**D**) T cells recruited to the mediastinal lymph node. (**E-G**) Total number of T cell subsets infiltrating the draining lymph node. T cells subsets were differentiated based on the following markers: naive ($CD3^+CD4^+CD44^-CD62L^{hi}$), effector memory ($CD3^+CD4^+CD44^{hi}CD62L^{lo}$) and central memory ($CD3^+CD4^+CD44^+CD62L^+$) T cells. Data represents three independent experiments. n= 4-6 mice per experiment. **P*<0.05, ***P*<0.001, and ****P*<0.0001 vs $CD28^{-/lox}Cre^{+/-}$ mice given oil.

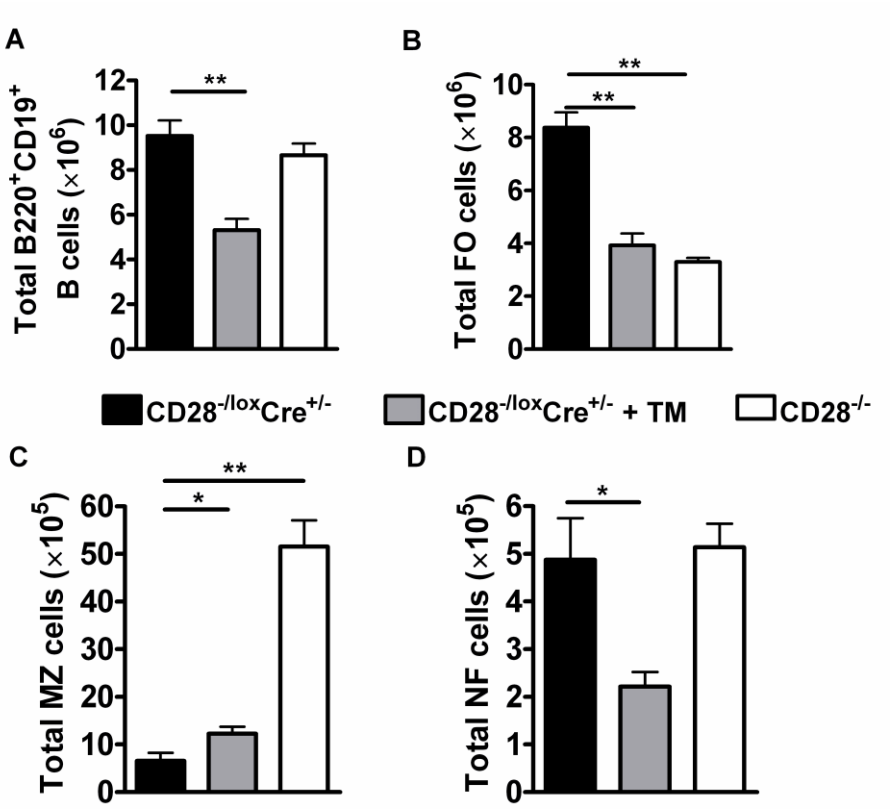

Figure 2.6: B cell development in the mediastinal lymph node is affected by CD28 deletion on CD4$^+$ T cells. Single cells suspension was prepared from mediastinal lymph nodes and cells stained for FACS. (**A**), Total numbers of CD19$^+$B220$^+$ B cells population recruited into the mediastinal lymph node. (**B-D**), Numbers of different B cell subsets recruited into the draining mediastinal lymph node. B cell subsets were differentiated based on the following markers: follicular B cells (FO, CD19$^+$B220$^+$CD21^{hi}CD23^{hi}), marginal zone B cells (MZ, CD19$^+$B220$^+$CD21^{hi}CD23^{lo}) and non-follicular B cell (NF, CD19$^+$B220$^+$CD21$^-$CD23$^-$).Data represents three independent experiments. n= 4-6 mice. *P<0.05 and **P<0.001 vs CD28$^{-/lox}$Cre$^{+/-}$ mice given oil.

2.4 DISCUSSION

The contribution of CD28 costimulation during recall of memory responses to infections has remained controversial despite numerous attempts to study it. Some studies have suggested that CD28 costimulation is not required for recall of protective memory responses to nematode infections [66,69]. Furthermore, infection of CD28$^{-/-}$ mice with the fungi *B. dermatitidis* showed that development of memory responses is CD28 independent [68]. In contrast, the absence of CD28 costimulation during recall of memory responses to *T. gondii* infection [70] and viral infections [71,72] inhibited the development of protective memory responses. The controversy surrounding the requirement of CD28 for recall of protective memory responses may depend on the pathogen itself. To address these questions for nematode infections, we utilised global CD28$^{-/-}$ mice, and more importantly a recently established conditional CD28 deleting mouse strain (CD28$^{-/lox}$Cre$^{+/-}$), where CD28 deletion is induced by oral administration of estrogen analogue tamoxifen [250]. The latter mouse model allowed for abrogation of CD28 costimulation after primary infection with *N. brasiliensis*, ensuring that priming of the immune response occurred in the presence of CD28 costimulation.

Primary infection in global $CD28^{-/-}$ mice with *N. brasiliensis* revealed that CD28 costimulation is essential for development of protective T cell immunity, as $CD28^{-/-}$ mice failed to expel adult worms in the small intestines efficiently, mainly caused by an impaired IL-13-driven Th2 cytokine response, known to play a crucial role in driving worm expulsion during primary infection with *N. brasiliensis* [145,195]. This outcome was also reproduced in anti-CD28 (E18) *in vivo* blocking experiments (our unpublished data) and is in agreement with previous studies by others, showing that interfering with CD28 costimulation during primary infection with nematodes impairs optimal development of Th2 cytokine responses [66,69]. Interestingly, analysis of the antibody responses showed reduced titres of both type 1 and type 2 antibody isotypes, indicating a general abrogation of humoral immunity in the absence of CD28 costimulation. This was further investigated during secondary infection. Contrastingly, $CD28^{-/-}$ mice inoculated with *H. polygyrus* primary infection mounted a sufficient humoral immune response [243]. Hence, the requirement of CD28 costimulation in driving development of humoral immunity seems to depend on parasites causing the infection. In fact, infection with *H. polygyrus* seems to be sufficient to induce polyclonal antibody responses even in the absence of CD28 costimulation, supporting the suggestion that parasites can trigger polyclonal B cells responses [253].

Abrogating CD28 costimulation during secondary infection in inducible CD28 deficient mice led to failure in mounting a protective memory response, strongly suggesting that CD28 costimulation is needed for efficient recall of protective immunity to *N. brasiliensis*. This conclusion is in contrast with previous findings using CTLA4-Ig to block the CD28 ligands CD80 and CD86 [66]. A possible explanation is that CTLA4-Ig exerts additional effects which may confound the situation. Thus, this fusion protein also prevents ligation of endogenous CTLA-4 expressed by regulatory and activated T-cells, which may partially counterbalance the desired effect. Failure of protective memory responses in inducible CD28 deficient mice was accompanied with striking reduced Th2 responses, including IL-4 and IL-13 in the lung draining lymph node. These results coincide with our current knowledge that CD28 costimulation enhances IL-4 receptor sensitivity and subsequently Th2 $CD4^+$ T cell differentiation [254,255], the latter being involved in mediating protective immunity against reinfection with *N. brasiliensis* by lung-resident$CD4^+$ T cells [256].

Mice deficient in CD28 signalling throughout the infection or prior secondary infection had reduced cellular numbers in the draining lymph nodes compared to littermate control mice. This may be due impaired recruitment of cells to the lymph nodes or the fact that CD28 is known to prevent apoptosis [53,242]. Previous studies have suggested a number of pathways governing the development of memory $CD4^+$ T cells. In a study by Hu and colleagues, memory $CD4^+$ T cells were shown to develop directly from effector $CD4^+$ T cells that reverted to a resting state suggesting a linear pathway for memory T cells generation [252]. However, other studies have suggested a more complex pathway for central memory T cell generation comprised of heterogeneous memory T cell populations [257]. Interestingly, the absolute numbers of $CD4^+$ T cells was reduced in mice which lost CD28 after primary *N. brasiliensis* infection, while there was no difference in absolute $CD4^+$ T cells numbers between $CD28^{-/-}$ mice and $CD28^{-/lox}Cre^{+/-}$ mice

given oil. This is probably due to a contribution of CD28 to the homeostasis of the CD4 T-cell compartment, which is less pronounced if CD28 is absent already during thymic differentiation. However, total numbers of effector $CD4^+$ T cells were markedly reduced in the absence of CD28 costimulation during secondary infection, regardless of whether CD28 was constitutively missing or deleted after priming. This resulted in reduced total numbers of central memory $CD4^+$ T cells in both $CD28^{-/lox}Cre^{+/-}$ mice given tamoxifen and $CD28^{-/-}$ mice.

Deficiency of CD28 costimulation also affected antibody responses as shown by reduced type 1 and type 2 antibody titres in mice deficient of CD28 costimulation. This data concurs with reduced total IgE titres observed in mice treated with CTLA4-Ig during reinfection with *N. brasiliensis* [66]. Hence, CD28 costimulation seems to be crucial for sustaining $CD4^+$ T cells dependent memory antibody responses. Cognate interaction between the antigen-specific T follicular helper cells (T_{FH}) and B cells in the lymphoid tissue is crucial for germinal center formation and optimal antibody responses including isotype switching [60]. In a study by Zaretsky and colleagues, IL-4 producing Th2 cells were shown to possess the capacity to differentiate into T_{FH} cells during immunisation with *S. mansoni* antigens [258]. This was further confirmed by a study by King and Mohrs that demonstrated that in a Th2 setting induced by infection with *H. polygyrus*, the majority of IL-4 producing $CD4^+$ T cells in the reactive lymph nodes co-express canonical T_{FH} cells markers and localised within the B cell follicles [259]. Our data showed that in the absence of CD28 costimulation, the expansion of T_{FH} cells expressing the canonical markers CXCR5 and ICOS was reduced during reinfection with *N. brasiliensis*. ICOS plays an essential role in maintaining the expression of CXCR5 on $CD4^+$ T cells during SRBC immunisation and enhances GC formation and antibody production [260]. Hence, we concluded that CD28 costimulation is important for development of T_{FH} cells in the reactive lymph nodes during secondary infection with the nematode *N. brasiliensis*. Furthermore, the deficiency of CD28 impacted on the development of B cells in the reactive lymph nodes, shown by diminished population of follicular B cells in infected global $CD28^{-/-}$ mice and as well as $CD28^{-/lox}Cre^{+/-}$ mice given tamoxifen after primary infection with *N. brasiliensis*. This can explain the diminished production of IL-4 in the reactive lymph nodes of CD28 mutant mouse strains and the observed abrogated humoral immunity. Together, these findings strongly demonstrate an important role played by CD28 costimulation during recall of protective memory responses to *N. brasiliensis* infection. CD28 costimulation seems to be required throughout the infection period to sustain the development of protective memory responses. These findings are in stark contrast to the normal development of protective memory responses exhibited by $CD28^{-/-}$ mice infected with *H. polygyrus* [69]. A recent study by Harvie and colleagues showed that the lungs are a crucial site harbouring protective immunity against *N. brasiliensis* reinfection [256]. The differences observed in the requirement of CD28 costimulation during recall of memory responses to *N. brasiliensis* and *H. polygyrus* may be due to different migration patterns of the parasites within the host. *H. polygyrus* is a completely enteric parasite while *N. brasiliensis* migrates via the lungs to the intestines.

In conclusion, our study clearly demonstrates the essential role played by CD28 costimulation during recall of protective memory responses to *N. brasiliensis* infection. CD28 costimulation was shown to confer protection against primary infection with *N. brasiliensis* using conventional $CD28^{-/-}$ mice. Failure to expel adult *N. brasiliensis* worms during secondary infection was associated with diminished Th2 cytokine responses and abrogated humoral immunity particularly the production of IgG1 and total IgE. Importantly, the deficiency of CD28 costimulation impaired recruitment of memory $CD4^{+}$ T cell sub-populations and expansion of T follicular helper cells crucial for providing help to follicular B cells.

CHAPTER 3

CHAPTER 3

IL-4/IL-13 responsive B cells are required for protection against acute schistosomiasis and down-modulating liver and gut inflammation.

H. Ndlovu[1#], N. Nieuwenhuizen[1], W. Horsnell[1], E. Hobeika[2,3] , M. Reth[2,3] and F. Brombacher[1*]

[1] International Center for Genetic Engineering and Biotechnology (ICGEB), Cape Town Component and Institute of Infectious Diseases and Molecular Medicine (IIDMM), Division of Immunology, University of Cape Town, Cape Town, South Africa.
[2] Centre for Biological Signaling Studies and Department of Molecular Immunology, Faculty of Biology, Albert-Ludwigs Universität Freiburg, Freiburg, Germany.
[3] Max Planck Institute for Immunobiology, Freiburg, Germany.

ABSTRACT

IL-4Rα dependent Th2 immune responses are indispensable for mounting host protective immunity against *S. mansoni* infection. B cells are known to play an important role in controlling *S. mansoni* infection but whether an ability to respond to IL-4/IL-13 impacts on this is unknown. To address this we used a novel mouse strain lacking IL-4Rα expression on B cells (*mb1*^{cre}IL-4Rα$^{-/lox}$). We found that B cell-specific IL-4Rα-deficient mice had heightened susceptibility to *S. mansoni* infection compared to IL-4Rα$^{-/lox}$ littermate control mice. Increased susceptibility was related to increased granuloma size; hepatocellular damage indicated by heightened serum AST levels and augmented gut inflammation. This was associated with reduced production of Th2 cytokines by total MLN cells from B cell-specific IL-4Rα-deficient mice after restimulation with SEA or α-CD3 *ex vivo*. Interestingly, abrogation of IL-4Rα signalling on B cells decreased secretion of both Th1 and Th2 cytokines by CD19^{+} B cells. Likewise, production of IL-4 and IL-13 by CD4^{+} T cells and recruitment of CXCR5^{+} T_{FH} cells and effector CD4^{+} T cells was impaired in mice lacking IL-4Rα expression on B cells. B cell-specific IL-4Rα$^{-/lox}$ mice failed to down-modulate granuloma formation and cytokine production during the chronic stages of infection. Generation of bone marrow chimera mice lacking IL-4 production specifically on B cells demonstrated that IL-4 produced by B cells is not involved in orchestrating host protective immunity, as indicated by normal antibody production, diminished granuloma formation, lack of iNOS producing cells in the liver granuloma and intact cytokine production by MLN cells after restimulation. These data demonstrate that B cell driven Th2 immunity plays a critical role in co-ordinating the induction of the immunological responses against acute schistosomiasis that are essential for host survival.

Keywords: IL-4Rα, B cells, *S. mansoni*, mb1^{cre}IL-4Rα$^{-/lox}$, mice.

3.1 Introduction

B cells are largely known for their capacity to produce antibodies in response to foreign antigens or pathogens. However, recent studies have expanded the functions of B cells during immune responses to pathogens. Activated B cells are competent antigen presenting cells (APCs) capable of internalising antigen through the B cell receptor (BCR), processing and presenting peptides to $CD4^+$ T cells [90,92,261]. Mice lacking the ability to present antigen due to the deletion of the MHC II molecule specifically on B cells failed to clear *Heligomosomoides polygyrus* (*Hp*) infection and exhibited impaired humoral and cellular immunity, demonstrating the importance of cognate interactions between B cells and $CD4^+$ T cells [90]. In fact, many studies have demonstrated the ability of antigen presenting B cells to assist in the expansion and differentiation of primary and memory $CD4^+$ T cells [89,261] and induce Th1 and Th2 cytokine production *in vivo* [87,94,96,97,262].

B cells have been shown to influence immunity to pathogens by producing a wide array of polarising cytokines such as IL-2, IL-4, IL-13, IFN-γ and IL-12 [93,104,105]. This has led to subdivision of B cells into three different effector B cell subsets that produce distinct cytokines, namely B effector 1 (Be1), B effector 2 (Be2) and regulatory B cells (Bregs) [106,107,263]. Be1 cells under the control of transcription factor T-bet produce IFN-γ, IL-12p40, TNF-α and IL-10 in response to cognate interaction with Th1 cells and antigen *in vitro* [104]. Such Be1 cells were detected *in vivo* during infection with a Th1 polarising parasite *Toxoplasma gondii* [104,106]. In contrast, development of Be2 cells that secrete IL-4, IL-2, IL-13, IL-6, IL-10 and TNF-α is dependent on IL-4, IL-4Rα and Th2 cells *in vitro* and infection with *Hp in vivo* has been shown to induce differentiation of Be2 cells [105,106]. Bregs are defined by their ability to produce IL-10 and TGF-β and are critical in immuno-suppression [263]. IL-10 producing B cells mediate protection against allergic airway inflammation [117], prevent colitis [118,119,120] and collagen-induced arthritis [121,122].

Cytokine producing effector B cells are crucial for the differentiation and expansion of effector Th2 cells during primary infection with *Hp* [90]. Although IL-4 producing Th2 cells are indispensable for host protection to *Hp* [264], chimeras lacking IL-4 production specifically in B cells cleared *Hp* infection, demonstrating that IL-4 producing B cells are not required for host protection and generation of protective memory Th2 cells [90]. In contrast, B cells producing IL-2 and TNF-α provided partial protection against *Hp* infection by regulating humoral and cellular immune responses in chimeric mice [90]. Moreover, the capacity of B cells to respond to IL-4 or IL-13 signalling via IL-4Rα expression is required for protective immunity to *Hp* challenge in chimeric mice [90]. The importance of IL-4/IL-13 responsive B cells in infection with *Schistosoma mansoni* (*S. mansoni*), a parasite that induces a highly polarised Th2 immune response, is yet to be determined.

Schistosomiasis is a parasitic disease caused by trematode parasites belonging to *Schistosoma* genus, affects more than 200 million people worldwide and is estimated to cause approximately 280 000 deaths per year in sub-Saharan Africa alone [203,265,266,267,268]. The disease is caused by parasite eggs that are lodged in the host's tissue where they elicit a highly polarised Th2 immune response which induces granulomatous inflammation [131,217]. Mice deficient in IL-4, IL-4/IL-13, IL-4Rα and STAT-6 quickly succumb to *S. mansoni* infection due to impaired granuloma formation, hepatocellular damage and augmented gut inflammation which leads to endotoxemia and septic shock [147,148,218,219]. Furthermore, studies from our laboratory have demonstrated that IL-4/IL-13 responsive macrophages [147], smooth muscle cells [188] and pan-T cells [190] are crucial for rendering host protection against *S. mansoni* infection and down-modulating tissue pathology.

During the chronic stage of *S. mansoni* infection, the marked Th2 immune response existing during the acute phase of infection is down-regulated alongside granuloma formation and tissue fibrosis [233,269]. Immuno-suppressive $CD8^+$ T cells [229], cross-regulation by cytokines produced by Th1 or Th2 cells [230,270,271] and FcR signalling on B cells [233] are all involved in modulating pathology during chronic Schistosomiasis. Recently, it has been demonstrated that interfering with IL-10R signalling results in augmented tissue damage during chronic *S. mansoni* infection due to loss of B cells in the liver [221], confirming the suppressive effect of IL-10 on Th2 cell differentiation during chronic schistosomiasis [220,222,231,235].

In this study, we investigated the role of IL-4/IL-13 responsive B cells in host protective immunity during acute schistosomiasis and the ability of these cells to down-modulate tissue pathology and T cell responsiveness during chronic *S. mansoni* infection, using transgenic mice (*mb1*^{cre}IL-4Rα$^{-/lox}$) lacking IL-4Rα expression on B cells. *Mb1*^{cre}IL-4Rα$^{-/lox}$ quickly succumbed to *S. mansoni* infection with similar kinetics to the highly susceptible IL-4Rα$^{-/-}$ mice. Increased susceptibility was associated with impaired production of Th2 cytokines, augmented granuloma formation; exacerbated liver damage indicated by high levels of serum AST, and severe gut inflammation. Mice deficient in IL-4Rα expression on B cells failed to down-modulate granuloma formation and exhibited mixed Th1, Th2 and Th17 cytokine responses at the later stages of chronic infection. Lastly, mice lacking IL-4 producing B cells developed normal Th2 immunity and had unaltered numbers of effector $CD4^+$ T cells and follicular B cells. Taken together, these findings demonstrate an important role for IL-4/IL-13 responsive B cells in conferring protection to *S. mansoni* infection and down-regulating tissue pathology.

3.2 Materials and Methods

Generation and genotyping of *mb1*^{cre}IL-4Rα$^{-/lox}$ Balb/c mice. *Mb1*^{cre} mice were intercrossed with IL-4Rα$^{lox/lox}$ Balb/c mice [147,191,272,273]. These mice were further mated with homozygous IL-4Rα$^{-/-}$ Balb/c mice [248] to generate hemizygous *mb1*^{cre}IL-4Rα$^{-/lox}$ mice. Hemizygous littermates (IL-4Rα$^{-/lox}$) expressing functional IL-4Rα were used as wild-type controls in all experiments. Mice were genotyped as described previously [147,248]. All mice were housed in specific pathogen-free barrier conditions in individually ventilated cages at the University of Cape Town biosafety level 2 animal facility. Experimental mice were age and sex matched and used between 8-12 weeks of age.

Ethics statement. This study was performed in strict accordance with the recommendations of the South African national guidelines and University of Cape Town practice of laboratory animal procedure. All mouse experiments were performed according to the protocols approved by the Animal Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (protocol number: 010/041). Efforts were made to minimise and reduce suffering of animals.

Live *S. mansoni* infection of mice. Mice were percutaneously infected with 100 live cercariae (acute infection) or with 30 live cercariae (chronic infection) of a Puerto Rican strain of *S. mansoni* obtained from infected *Biomphalaria glabrata* (a generous gift from Adrian Mountford, York, UK). The mice were monitored weekly until 7 weeks post-infection.

Pulmonary *S. mansoni* eggs model. Synchronous *S. mansoni* egg-challenge was conducted as previously described [274]. Briefly, mice were sensitised to schistosome eggs by intraperitoneal injection of 2 500 eggs. Mice were subsequently challenged 14 days later by intravenous injection of 2 500 eggs and killed at day 7 and 14 post-challenge.

Antibodies and flow cytometry. The following antibodies comprising the B cell antibody panel were used: B220-V500, CD19-PerCP Cy5.5, CD23-PE, CD21-APC, CD24-PE Cy7, CD80-V450, MHCII-FITC and IgM-Biotin (BD Bioscience, Erembodegem, Belgium). T cells panel consisted of the following antibodies: CD4-PerCP, CD3-AlexaFluor 700, CD62L-V500, CD44-FITC, CD28-PE, CXCR5-V450 and CD278-Biotin (BD Bioscience, Erembodegem, Belgium). Cells were acquired on a FACS Fortessa machine (BD Immunocytometry system, San Jose, CA, USA) and data was analyzed using Flowjo software (Treestar, Ashland, OR, USA). Macrophage activation profile was analysed in CD11b^{+}MHCII^{+} macrophages by flow cytometry. Classically activated macrophages were detected by staining for intracellular expression of iNOS using rabbit anti-mouse iNOS antibody (Abcam) with goat anti-rabbit PE (Abcam). Alternatively activated macrophages were detected by detecting Arginase 1 and Ym-1-Biotin using goat anti-mouse arginase (Santa Cruz Biotechnology) with donkey anti-goat PE (Abcam) and Strep-APC

respectively. Staining specificity was verified by appropriate isotype-matched antibody controls and compensation performed with single-stain samples before acquiring the multi-coloured samples. Acquisition was performed using a FACSCalibur (BD Immunocytometry Systems).

Intracellular cytokine staining. For detection of intracellular cytokines, MLN cells from *S. mansoni* infected mice or MST from *S. mansoni* eggs injected mice were plated at 1×10^6 cells/well and stimulated at 37ºC for 4 hours with 50 ng/ml phorbol (myristate acetate), 250 ng/ml ionomycin and 200 µM monensin in IMDM/10% FCS (all purchased from Sigma-Aldrich). Cells were stained with extracellular markers (CD4 Biotin-APC, B220 FITC or CD19 PercP), fixed for 30 min on ice in 2% (w/v) paraformaldehyde and permeabilised with 0.5% saponin buffer and stained with PE-labelled anti-mouse IL-4, IL-5, IL-10 and IFN-γ for 15 min. Acquisition was performed using a FACSCalibur (BD Immunocytometry Systems, San Jose, CA, USA) and data were analysed using FlowJo software (Treestar, Ashland, OR, USA).

Cell preparation and *ex vivo* restimulation. Single cell suspensions were prepared by pressing the draining lymph nodes through 70 µM cell-strainers. Cells were resuspended in complete IMDM (Gibco) supplemented with 10% FCS (Gibco) and penicillin and streptomycin (100 U/ml and 100 µg/ml, Gibco). The cells were cultured at 2×10^6 cells/ml in 48-well plates coated with α-CD3 (100 µg/ml) or soluble egg antigen (SEA, 20 µg/ml) and incubated at 37ºC in a humidified atmosphere containing 5% CO_2. Supernatants were collected after 72 h and cytokines were measured by ELISA. Quantities of IL-4, IL-10 and IL-13 were measured by sandwich ELISA as previously described [248].

Preparation of cells from liver tissue. A method described by Metwali and colleagues was used to isolate granuloma associated cells [275]. Briefly, infected livers were finely chopped and then suspended in complete IMDM (Gibco) supplemented with 5% collagenase and the tissue suspension was incubated at 37ºC for one hour. The digested tissue was centrifuged at 5 000 ×g and the sediment was washed twice with ice-cold PBS. The dispersed granulomas were further disrupted by repeated suction and expulsion through a 20 ml syringe. The cells were washed and spun again and then viability was determined by staining cells with trypan blue.

Enzyme Linked Immunosorbent Assays (ELISAs). Cytokines in supernatant were measured by sandwich ELISA as previously described [248]. For antibody ELISAs, blood was collected in serum separator tubes (BD Bioscience, San Diego, CA) and serum was separated by centrifugation at 8 000 ×g for 10 min at 4ºC. Titres of SEA-specific IgG1, IgG2a, IgG2b and total IgE were determined as previously described [248].

Hydroxyproline assay. Hydroxyproline content as a measure of collagen production was determined using a modified protocol [276]. Briefly, weighed liver samples were hydrolysed and added to a 40 mg Dowex/Norit mixture. The supernatants were neutralised with 1% phenolphthalein and titrated against 10 M NaOH. An aliquot was mixed with isopropanol and added to chloramine-T/citrate buffer solution (pH 6.5). Erlich's reagent was added and absorbance was read at 570 nm. Hydroxyproline levels were calculated using 4-hydroxy-L-proline (Calbiochem) as a standard, and results were expressed as µmoles hydroxyproline per weight of tissue that contained 10^4 eggs.

Histology. Liver and gut samples were fixed in 4% (v/v) formaldehyde in phosphate buffered saline, embedded in wax and processed. Sections (5-7 µm) were stained with hematoxylin and eosin (H&E) and analine blue solution (CAB) and counterstained with Wegert's hematoxylin for collagen staining. Micrographs of liver granuloma were captured using a Nikon 5.0 mega pixel colour digital camera (DCT DS-SMc).The diameter of each granuloma containing a single egg was measured with the ImageJ 1.34 software. An average of 25 granulomas per mouse was included in the analyses.

Statistics. Statistical analysis was conducted using GraphPad Prism 4 software. Data was calculated as mean ± SD. Statistical significant was determined using the unpaired Student's *t* test or 2-way ANOVA with Bonferroni's post test, defining differences to IL-4Rα$^{-/lox}$ mice as significant (*,$p \leq 0.05$; **,$p \leq 0.01$; ***,$p \leq 0.001$). (Prism software; http://www.prism-software.com).

3.3 Results

B cell-specific IL-4Rα deficient mice are highly susceptible to acute Schistosomiasis

Studies conducted in mice deficient in B cells (µMT) have demonstrated a critical role for B cells in down-regulating tissue pathology and conferring host protection during the chronic stage of *S. mansoni* infection [232,233]. Furthermore, mice lacking expression of the FcγR chain displayed augmented granuloma formation during both the acute and chronic stages of infection [233]. Therefore, these studies were essential in elucidating the role of B cells during the development of host protective immune responses against *S. mansoni* infection. However, the contribution of B cells responding to IL-4/IL-13 signalling via IL-4Rα in host survival and immunity during schistosomiasis is unknown.

In order to determine the role of IL-4/IL-13 responsive B cells during acute *S. mansoni* infection, *mb1*^{cre}IL-4Rα$^{-/lox}$ Balb/c mice were infected with 100 live *S. mansoni* cercariae and mortality monitored over a 14 weeks period (Fig 3.1A). *Mb1*^{cre}IL-4Rα$^{-/lox}$ mice quickly succumbed to *S. mansoni* infection by 10 weeks post-infection, similarly to the highly susceptible IL-4Rα$^{-/-}$ mice (Fig 3.1A). In contrast, IL-4Rα$^{-/lox}$ littermate control mice displayed greater resistance to *S. mansoni* infection, with 40% of the mice surviving until 10 weeks post-infection (Fig 3.1A). IL-

4Rα$^{-/-}$ mice are known to succumb to infection due to severe cachexia that coincides with the onset of egg-laying by the parasites [147,190]. As expected, IL-4Rα$^{-/-}$ began to drastically lose weight after 6 weeks post-infection and some mice had lost 20% of their body weight by the time of death (Fig 3.1B). However, *mb1*^{cre}IL-4R$^{-/lox}$ and IL-4Rα$^{-/lox}$ mice displayed similar kinetics for weight loss up until 9 weeks post-infection, when littermate control mice recovered some of their body weight (Fig 3.1B). Together, these data demonstrate an important role for IL-4/IL-13 responsive B cells in host survival during acute schistosomiasis.

To investigate the impact of *S. mansoni* infection in organ pathology, we conducted post-mortems on infected mice. There was no significant difference in spleen (Fig 3.1C) and liver (Fig 3.1D) weight between B cell-specific IL-4Rα deficient mice and littermate control mice. Conversely, IL-4Rα$^{-/-}$ mice had significantly reduced spleen (Fig 3.1C) and liver (Fig 3.1D) weight compared to the littermate control mice, indicating severe multiple organ defects. Furthermore, examination of autopsy images indicated extensive haemorrhaging in the small intestines of IL-4Rα$^{-/-}$ mice compared to littermate control mice that exhibited no gut inflammation (Fig 3.1E). Interestingly, *mb1*^{cre}IL-4Rα$^{-/lox}$ mice developed slight haemorrhaging in the gut lumen that was accompanied by colitis-type pathology (Fig 3.1E). Thus, these data suggest that hepatosplenomegaly and gut inflammation contribute to the increased susceptibility observed in B cell-specific IL-4Rα deficient mice.

Reduced Th2 responses in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice during *S. mansoni* infection

To investigate the cellular mechanism of susceptibility in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice during acute Schistosomiasis, we analysed cytokine production by total mesenteric lymph node (MLN) cells restimulated with either SEA or α-CD3 *ex vivo*. The abrogation of IL-4Rα expression on B cells significantly impaired the production of the Th2 cytokines IL-4, IL-5 and IL-10 by MLN cells after stimulation with SEA compared to the littermate control mice (Fig 3.2A). However, production of Th1 and Th17 cytokines by MLN cells from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice following antigen-specific restimulation was not altered compared to littermate control mice (Fig 3.2A). In contrast, IL-4Rα$^{-/-}$ mice displayed a shift towards Th1 immune responses, characterised by augmented IFN-γ production by MLN cells stimulated with SEA and concomitant reduction in Th2 cytokine production (Fig 3.2A). Mitogenic restimulation of MLN cells from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice revealed diminished Th2 cytokine production, with the exception of IL-4, compared to littermate control mice (Fig 3.2B). Finally, a predominant Th1 and Th17 cytokine profile was produced by mitogen restimulated MLN cells from IL-4R$^{-/-}$ mice compared with littermate control mice (Fig 3.2B). Therefore, these data demonstrate that IL-4/IL-13 responsive B cells are crucial for development of Th2 immunity during *S. mansoni* infection in mice.

To explore the impact of IL-4Rα deficiency on B cells on the differentiation and cytokine production by CD4^{+} T cells during infection, single cell suspensions were prepared from MLN

and cells were stained for flow cytometric analysis. Interference with IL-4Rα expression on B cells did not affect cellular infiltration into the draining lymph node (data not shown). However, global abrogation of IL-4Rα resulted in reduced infiltration of cells into the MLN compared to the littermate control mice (data not shown). There was no significant difference in the absolute number of $CD3^+CD4^+$ T cells present in the MLN of all mutant mouse strains (Fig 3.2C). However, the lack of IL-4Rα expression on B cells resulted in significantly lower numbers of $CXCR5^+$ T_{FH} cells and effector $CD4^+$ T cells ($CD4^+CD44^{hi}D62L^{lo}$) compared to control mice (Fig 3.2D & E). Similarly, global IL-4Rα deficient mice exhibited reduced numbers of $CXCR5^+$ T_{FH} cells and effector $CD4^+$ T cells compared to littermate control mice (Fig 3.2D & E). Examination of intracellular cytokine production by $CD4^+$ T cells restimulated with PMA/Ionomycin *ex vivo* revealed that abrogation of IL-4Rα expression on B cells results in reduced production of Th2 cytokines IL-4 and IL-13, while IFN-γ production was not altered compared to control mice (Fig 3.2F, G & H). Likewise, $CD4^+$ T cells from IL-$4R\alpha^{-/-}$ mice failed to produce Th2 cytokines IL-4 (Fig 3.2F) and IL-13 (Fig 3.2G) after *ex vivo* restimulation with PMA/Ionomycin compared to littermate control mice. Production of the Th1 cytokine IFN-γ was also reduced in $CD4^+$ T cells from IL-$4R\alpha^{-/-}$ mice compared to littermate control mice, suggesting overall inhibition of T cell responses. In summary, IL-4Rα expression on B cells is crucial for differentiation of $CD4^+$ T cells and generation of $CD4^+$ Th2 immunity.

Reduced type 2 antibody responses and B cell-specific cytokine production in *S. mansoni* infected *mb1*^{cre}IL-4R$\alpha^{-/lox}$ mice

The ability of B cells to respond to IL-4 signalling is crucial for isotype switching towards type 2 antibody isotypes IgG1 and IgE during *S. mansoni* infection [253]. To assess the impact of IL-4Rα deficiency in B cells on antibody production, we analysed sera from infected mice by ELISA. As expected, sera from infected B cell-specific IL-4Rα deficient mice contained low levels of SEA-specific IgG1 and total IgE antibody titers, similar to global IL-$4R\alpha^{-/-}$ mice (Fig 3.3A). In contrast, littermate control mice had higher type 2 antibody titers (IgG1 and total IgE), accompanied by concomitant reduced levels of antigen-specific type 1 antibody (IgG2a) titers (Fig 3.3A). Therefore, these data confirm a pivotal role for IL-4/IL-13 responsive B cells in the generation of type 2 antibody responses during *S. mansoni* infection in mice.

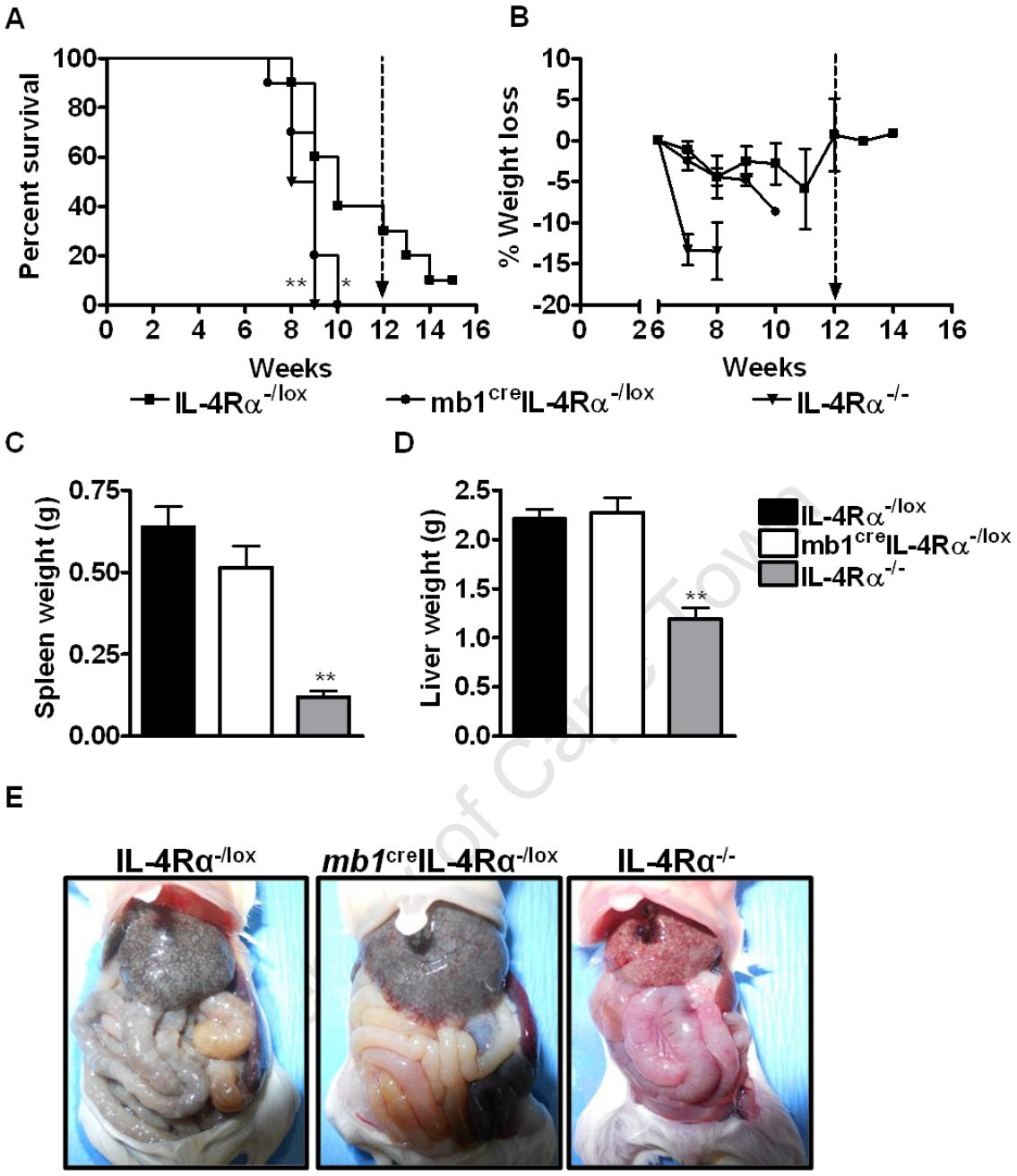

Figure 3.1: IL-4/IL-13 responsive B cells are required for protection against acute schistosomiasis in mice. IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 100 live *S. mansoni* cercariae and survival was monitored over a 14 weeks period. (**A**) Survival kinetics of mice infected percutaneously with 100 cercariae. **(B)** Percent body weight loss monitored on a weekly basis. (**C**) Spleen weight measured during autopsy. (**D**) Liver weight measured during autopsy. (**E**) Post-mortem images depicting pathology in infected mice. Data represents two independent experiments (n = 8-10). Survival curves were compared using Logrank test. *p< 0.05 and **p< 0.01 vs IL-4Rα$^{-/lox}$ mice.

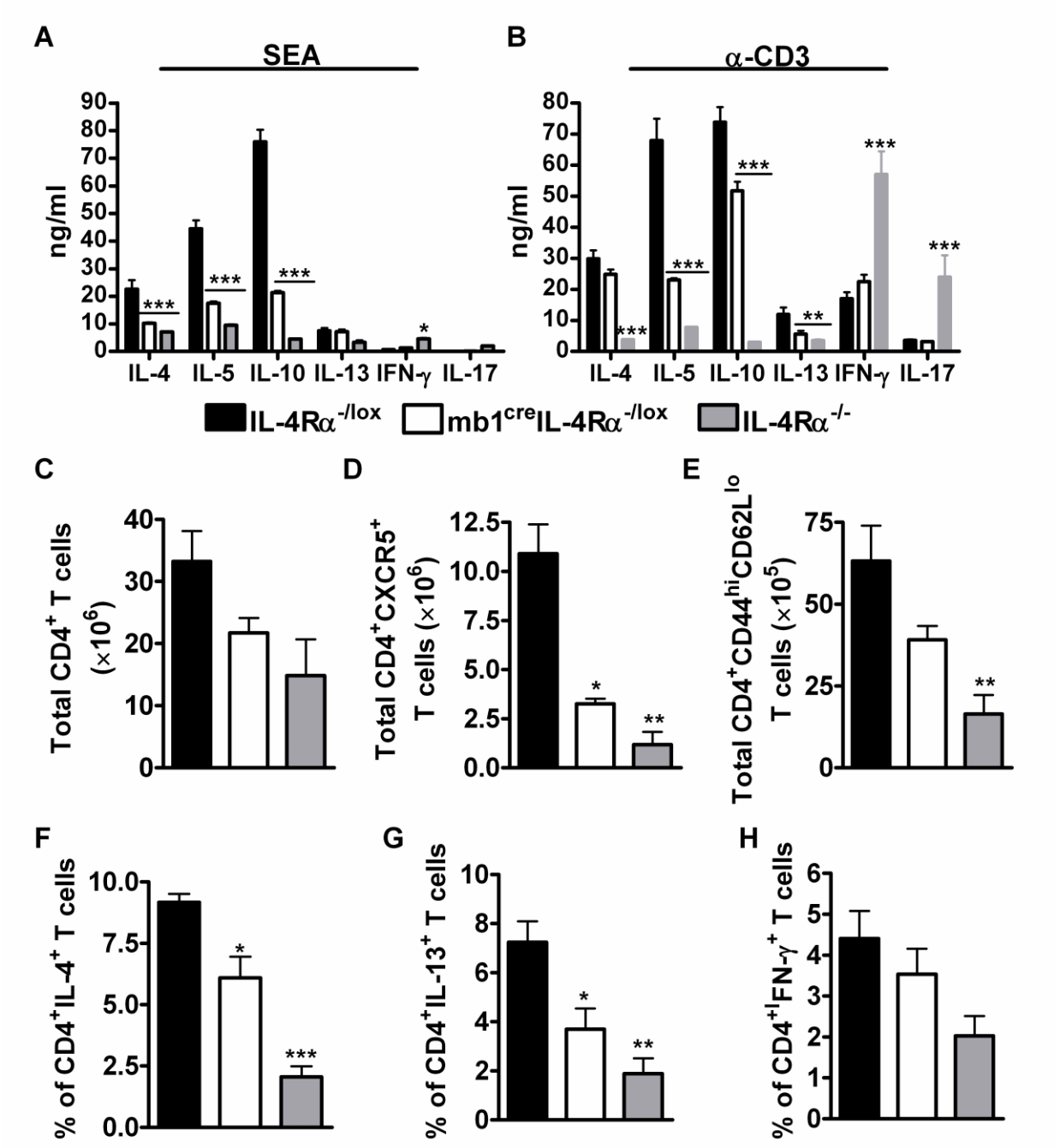

Figure 3.2: Reduced Th2 immunity in B cell-specific IL-4Rα deficient mice.
IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 100 live *S. mansoni* cercariae and analysed 7 weeks post-infection. (**A-B**) Cytokine production by mesenteric lymph node cells restimulated with either SEA or α-CD3 (mean ± SEM). Single cell suspension was prepared from mesenteric lymph node (MLN) and cells were stained for flow cytometric analysis. (**C**) Recruitment of CD3^{+}CD4^{+} T cells into the secondary lymphoid tissue. (**D-E**) Expansion of CXCR5^{+} T_{FH} cells and effector CD4^{+} T cells in the MLN. (**F-H**) Intracellular cytokine detection after restimulation of MLN cells with 25 ng/ml PMA and 1 µg/ml ionomycin *in vitro*. Data represents 3 independent experiments. n = 4-6 mice. *p<0.05, **p<, 0.01 and ***p< 0.001 vs IL-4Rα$^{-/lox}$ mice.

To investigate the importance of IL-4Rα signalling in the differentiation of B cell subsets during *S. mansoni* infection, expression of CD21 and CD23 was analysed by flow cytometry. There was no difference in the absolute number of CD19^{+}B220^{+} B cells draining into the MLN (Fig 3B).

However, the absolute number of follicular B cells (FO, $B220^{+}CD21^{+}CD23^{+}$) was significantly reduced in both *mb1*^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/-}$ mice compared to littermate control mice (Fig 3.3C). Simultaneously, the absolute number of marginal zone (MZ, $B220^{+}CD21^{+}CD23^{-}$) B cells was increased in B cell-specific IL-4Rα deficient mice while it remained similar between IL-4Rα$^{-/-}$ and littermate control mice (Fig 3.3D). Interestingly, expression of activation markers MHC II and CD86 was reduced in both *mb1*^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/-}$ mice compared to littermate control mice (data not shown). These data suggests that B cell-specific IL-4Rα expression is essential for B cell differentiation and expression of activation markers during *S. mansoni* infection.

B cells have been shown to produce cytokines in response to antigen-specific stimulation or *in vivo* during infection [106]. To explore the ability of IL-4Rα deficient B cells to produce cytokines during *S. mansoni* infection, single cell suspensions were prepared from MLN and cells were restimulated with PMA/Ionomycin before intracellular cytokine detection by flow cytometry. The percentage of $CD19^{+}$ B cells producing Th2 cytokines IL-4 and IL-13 was significantly reduced in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice (Fig 3.3E). Intriguingly, the percentage of IFN-γ producing B cells were also significantly reduced in B cell-specific IL-4Rα deficient mice compared to littermate control mice. Likewise, the percentage of $CD19^{+}$ B cells producing IL-4 and IL-13 was significantly reduced in IL-4Rα$^{-/-}$ mice, accompanied by a simultaneous reduction in the percentage of $CD19^{+}$ B cells producing IFN-γ and TNF-α compared to control mice (Fig 3.3E). Therefore, these data suggests that there is a general reduction in cytokine production by $CD19^{+}$ B cells in the absence of IL-4Rα expression during *S. mansoni* infection.

Augmented liver pathology in *S. mansoni* infected *mb1*^{cre}IL-4Rα$^{-/lox}$ mice

Prior studies from our laboratory have demonstrated that liver pathology caused by uncontrolled granuloma formation, increased hepatocellular damage and tissue fibrosis is a major factor contributing to susceptibility to acute schistosomiasis [147,190]. In order to investigate the role played by IL-4/IL-13 responsive B cells during liver pathology caused by trapped *S. mansoni* eggs, liver tissue was examined by histology and serum AST levels were assayed. Liver inflammatory pathology was increased in B cell-specific IL-4Rα deficient mice, which had increased granuloma size (Fig 3.4A), unaltered liver fibrosis, measured as hydroxyproline concentration (Fig 3.4B) and significantly elevated levels of serum AST (Fig 3.4C), indicating increased hepatocellular damage compared with littermate control mice. In contrast, IL-4Rα$^{-/-}$ mice had significantly impaired granuloma formation (Fig 3.4A), slightly reduced fibrosis (Fig 3.4B) and augmented hepatocellular damage (Fig 3.4C). In *mb1*^{cre}IL-4Rα$^{-/lox}$ mice, liver pathology was associated with diminished Th1 and Th2 cytokine production by liver granuloma-associated cells stimulated with either SEA or α-CD3 compared with littermate control mice (Fig 3.4D). Liver granuloma-associated cells from IL-4Rα$^{-/-}$ mice restimulated with α-CD3 produced high levels of IL-6 and IFN-γ while the levels of IL-4 and IL-5 were down-regulated compared with

IL-4Rα$^{-/lox}$ mice (Fig 3.4D). Histological examination of H&E stained liver sections confirmed prior data, where granuloma formation was augmented in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice (Fig 3.4E) and reduced in IL-4Rα$^{-/-}$ mice compared to littermate control mice. Interestingly, liver granuloma-associated cells from IL-4Rα$^{-/-}$ mice stained positive for iNOS (Fig 3.4E), a marker for classically activated macrophages [147]. In contrast, there were a few iNOS positive cells in granulomas from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice while iNOS positive cells were not detected in granulomas from infected littermate control mice (Fig 3.4E). Therefore, these data suggest that IL-4Rα expression by B cells is crucial for down-modulating liver pathology during *S. mansoni* infection in mice.

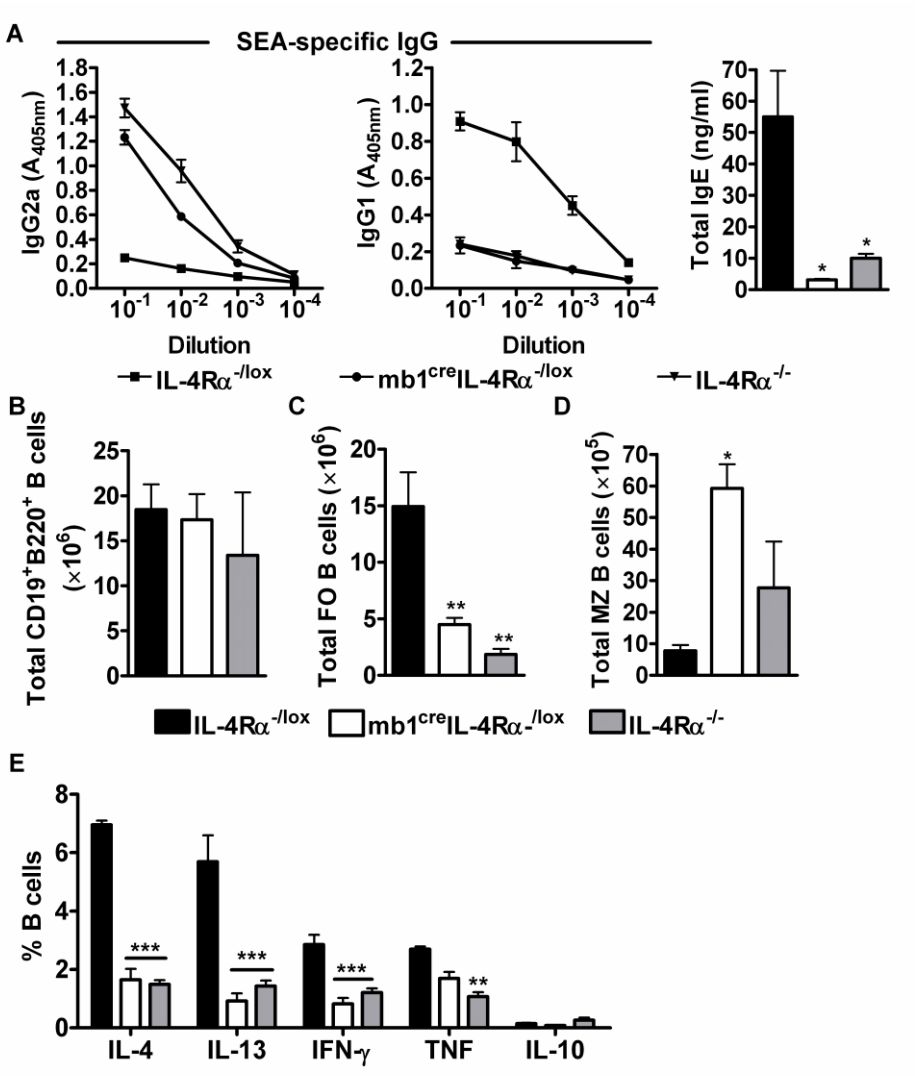

Figure 3.3: Impaired type 2 antibody responses in B cell-specific IL-4R deficient mice.
IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 100 live *S. mansoni* cercariae and analysed 7 weeks post-infection. (**A**) Antigen-specific immunoglobulin (IgG) and total IgE titers were detected by ELISA. Single cell suspensions were prepared from mesenteric lymph nodes (MLN) and cells were stained for flow cytometry. (**B-D**) Recruitment of CD19^{+}B220^{+} B cells, follicular B cells (FO, B220^{+}CD21^{+}CD23^{+}) and marginal zone B cells (MZ, B220^{+}CD21^{+}CD23^{-}) into the secondary lymphoid tissue. (**E**) Analysis of intracellular cytokine production by CD19^{+} B cells after restimulation of total MLN cells with 25 ng/ml PMA and 1 µg/ml ionomycin *in vitro*. Data represents 3 independent experiments. n = 4-6 mice. *p<0.05, **p<, 0.01 and ***p< 0.001 vs IL-4Rα$^{-/lox}$ mice.

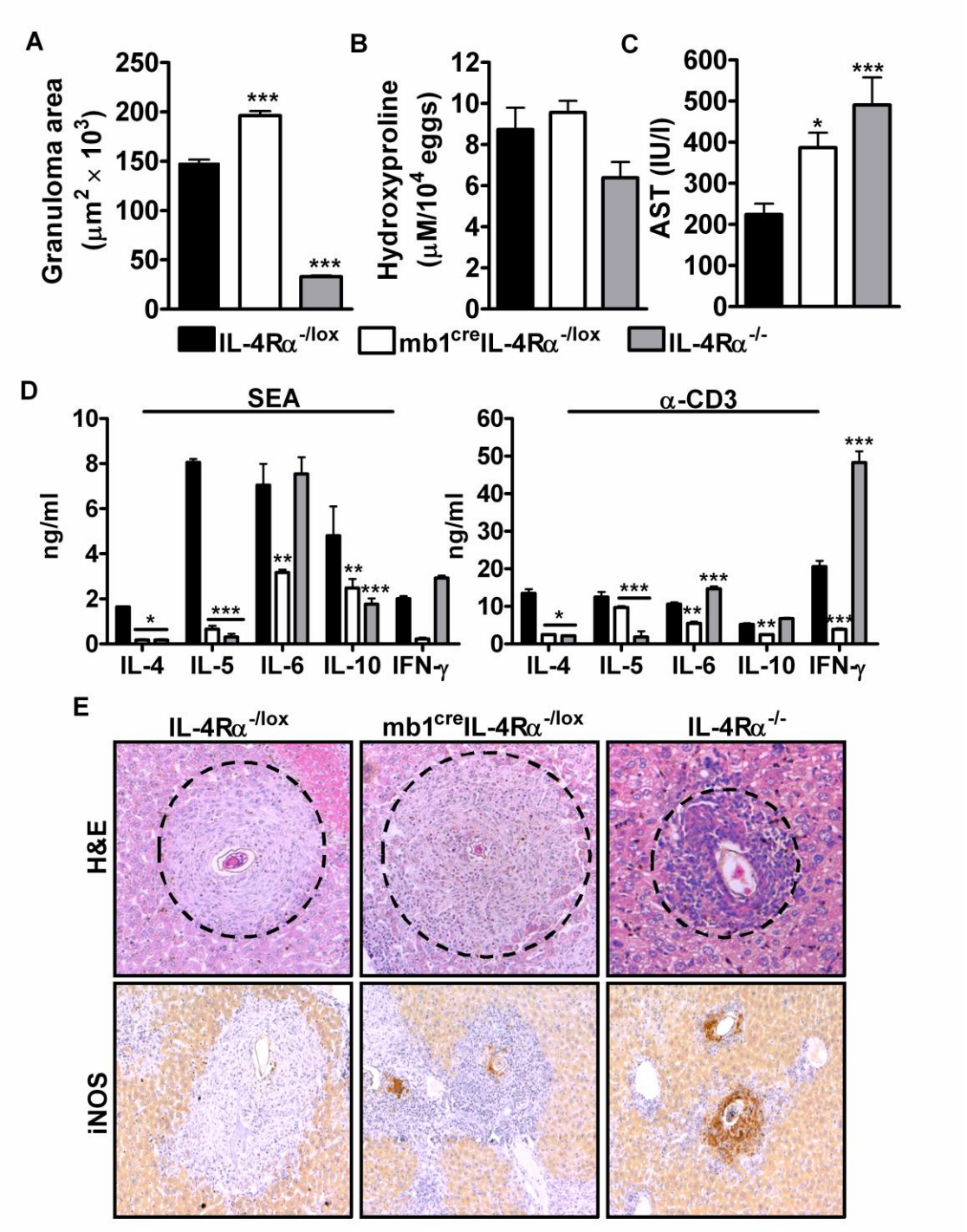

Figure 3.4: IL-4Rα responsive B cells are required to down-regulate liver granuloma formation and hepatocellular damage.
IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 100 *S. mansoni* cercariae and analysed 7 weeks post-infection. (**A**) Granuloma area surrounding eggs quantified by microscopic analysis on H&E stained sections. Twenty to 30 granulomas per mouse were included in the measurement of granuloma area. (**B**) Liver fibrosis measured as hydroxyproline normalised to egg numbers. (**C**) Hepatocellular damage quantified as serum aspartate transaminase (AST) levels. (**D**) Cytokine production by total liver cells restimulated with either SEA or α-CD3. (**E**) Formalin-fixed liver sections (100×) stained with H&E for morphological analysis or iNOS for classical macrophages surrounding the eggs. Data is representative of 3 independent experiments. n= 4-6 mice. *p< 0.05, **p< 0.01 and ***p< 0.001 vs IL-4Rα$^{-/lox}$.

Increased gut inflammation in *S. mansoni* infected *mb1*^{cre}IL-4Rα$^{-/lox}$ mice

Mice that are highly susceptible to *S. mansoni* infection suffer from severe gut inflammation characterised by haemorrhaging in the gut, which ultimately results in endotoxemia [147,148,218,222]. To investigate whether mice lacking IL-4Rα expression on B cells can control gut integrity during infection, we examined gut pathology by histology and quantified hydroxyproline content. There was no significant difference in the quantity of gut hydroxyproline and tissue egg numbers between the different mutant mouse strains (Fig 3.5A & B). However, examination of H&E stained gut section from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice revealed intensive gut inflammation, similar to that observed in the highly susceptible IL-4Rα$^{-/-}$ mice (Fig 3.5C). Furthermore, there were an increased number of inflammatory cells surrounding *S. mansoni* eggs compared to littermate control mice that exhibited small gut granulomas (Fig 3.5C). Together, these data suggest that B cell-specific IL-4Rα expression is necessary to down-modulate gut inflammation during acute schistosomiasis in mice.

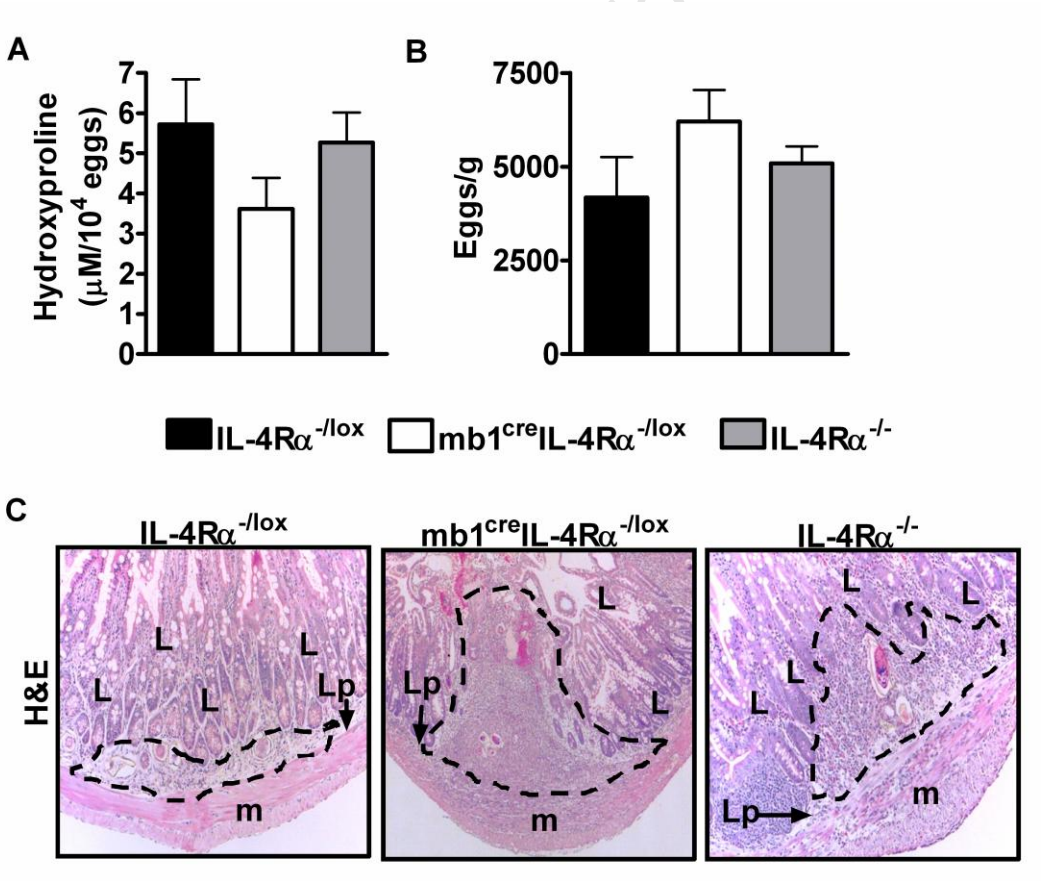

Figure 3.5: IL-4Rα dependent B cell responses contribute to controlling gut pathology during acute schistosomiasis.
IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 100 *S. mansoni* cercariae and analysed 7 weeks post-infection. (**A**) Intestinal fibrosis measured as hydroxyproline concentration normalised to tissue egg numbers (mean ± SEM, n= 4-6). (**B**) Intestinal eggs numbers normalised to tissue weight (mean ± SEM, n= 4-6). (**C**) Increased inflammation shown in H&E stained sections of the small intestine. L, lacteal spaces; m, muscularis layer and Lp, lamina propria. Original magnification, ×100. Data represents three independent experiments.

Macrophages display a classical activation profile in *S. mansoni* infected B cell-specific IL-4Rα deficient mice

Alternatively activated macrophages are crucial for mediating protection against acute schistosomiasis and limit tissue destruction caused by eggs trapped in the host tissue [147]. To investigate the activation profile of macrophages in the liver from infected *mb1*^{cre}IL-4Rα$^{-/lox}$, we prepared single cell suspensions from liver tissue and stained the cells for flow cytometric analysis. There was an increased proportion of iNOS positive CD11b^{+}MHCII^{+} macrophages in both B cell-specific IL-4Rα deficient mice and global IL-4Rα knock-out mice compared to littermate control mice, suggesting a shift towards classical activation (Fig 3.6A). Furthermore, iNOS expression was significantly increased in macrophages from *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice compared to control mice (Fig 3.6B). Conversely, the proportions of Ym-1 positive CD11b^{+}MHCII^{+} macrophages were reduced in both *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice compared to littermate control mice (Fig 3.6C). Analysis of Ym-1 expression levels confirmed down-regulation of Ym-1 production in macrophages from B cell-specific IL-4Rα deficient mice and IL-4Rα$^{-/-}$ mice compared to littermate control mice (Fig 3.6D). Therefore, these data suggest that either B cells expressing IL-4Rα interact directly with macrophages, influencing their activation profile, or that the prevailing cytokine microenvironment influences macrophage activation status during *S. mansoni* infection.

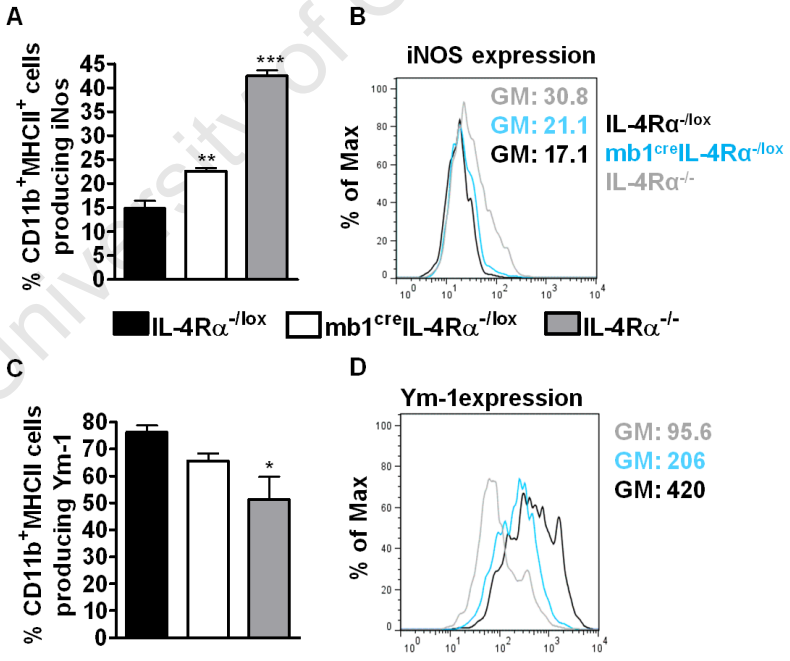

Figure 3.6: Reduced expression of alternative activated macrophage markers in B cell-specific IL-4Rα deficient mice.
Single cell suspensions were prepared from liver tissue and cells were stained for flow cytometry. (**A**) Proportions of CD11b^{+}MHCII^{+} macrophages expressing iNOS in the liver granulomas. (**B**) Histogram showing the expression of iNOS by macrophages. (**C**) Proportions of CD11b^{+}MHCII^{+} macrophages expressing alternative activation marker Ym-1. (**D**) Histogram showing the expression of Ym-1 by macrophages. Data represents two independent experiments. **p< 0.01, ***p<0.001 vs IL-4Rα$^{-/lox}$ mice.

IL-4 responsiveness by B cells is crucial for host survival during schistosomiasis

Mice deficient in B cells display heightened susceptibility at the chronic stage of *S. mansoni* infection [233]. To investigate whether *mb1*^{cre}IL-4Rα$^{-/lox}$ mice are more susceptible to *S. mansoni* infection than B cell knock-out mice (μMT), we conducted a mortality study where *mb1*^{cre}IL-4Rα$^{-/lox}$, μMT and IL-4Rα$^{-/lox}$ mice were infected with 100 live *S. mansoni* cercariae and mortality was monitored over a 13 week period. *Mb1*^{cre}IL-4Rα$^{-/lox}$ mice began to die at 7 weeks post-infection and they had all succumbed to infection by 10 weeks post-infection compared to littermate control mice that had 50% survival at the same time point (Fig 3.7A). In contrast, B cell deficient mice displayed delayed susceptibility, with mice surviving the acute phase of infection (Fig 3.7A). However, during the chronic phase of infection, B cell knock-out mice had a drastic increase in mortality rate, with 50% of the mice succumbing to infection within the same week (Fig 3.7A). Together, these data suggest that the ability of B cells to respond to IL-4/IL-13 signalling and not the general lack of B cells is essential for host survival during *S. mansoni* infection.

Liver granuloma formation was compared between *mb1*^{cre}IL-4Rα$^{-/lox}$ mice and μMT mice at 7 weeks post *S. mansoni* infection. As expected, B cell-specific IL-4Rα deficient mice had augmented granuloma size compared to littermate control mice (Fig 3.7B). However, the granulomas from B cell knock-out mice were larger than the ones from B cell-specific IL-4Rα deficient mice (Fig 3.7B). In fact, B cell knock-out mice developed granulomas that were almost twice the size of those from littermate IL-4Rα$^{-/lox}$ mice (Fig 3.7B). Livers from μMT mice contained almost double the concentration of hydroxyproline than both the *mb1*^{cre}IL-4Rα$^{-/lox}$ and littermate control mice during the acute phase of infection, indicating increased hepatic fibrosis (Fig 3.7C). These data suggest that the lack of B cells results in uncontrolled liver pathology in mice.

To investigate whether the lack of IL-4/IL-13 responsive B cells influences cellular immunity, we restimulated MLN cells from infected mice with either SEA or α-CD3 *ex vivo* and measured cytokine production by ELISA. In accordance with prior data, cells from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice failed to produce Th2 cytokines IL-4, IL-5 and IL-10 in response to antigen-specific stimuli compared to littermate control mice (Fig 3.7D). Similarly, cells from μMT mice stimulated with SEA failed to produce IL-4 and IL-10 although the levels of IL-5 were the same as that in littermate control mice (Fig 3.7D). Conversely, mitogenic stimulation of cells from μMT mice triggered a substantial release of IL-10 and IFN-γ while the production of IL-4 was diminished compared to littermate control mice (Fig 3.7D). Finally, in comparison to littermate control mice, cells from B cell-specific IL-4Rα deficient mice showed defects in the production of IL-4 and IL-5 while the production of IL-10 and IFN-γ was unaltered after restimulation with α-CD3 (Fig 3.7D). These data suggest that abrogating IL-4Rα expression on B cells impairs the development of Th2 immunity during *S. mansoni* infection in mice.

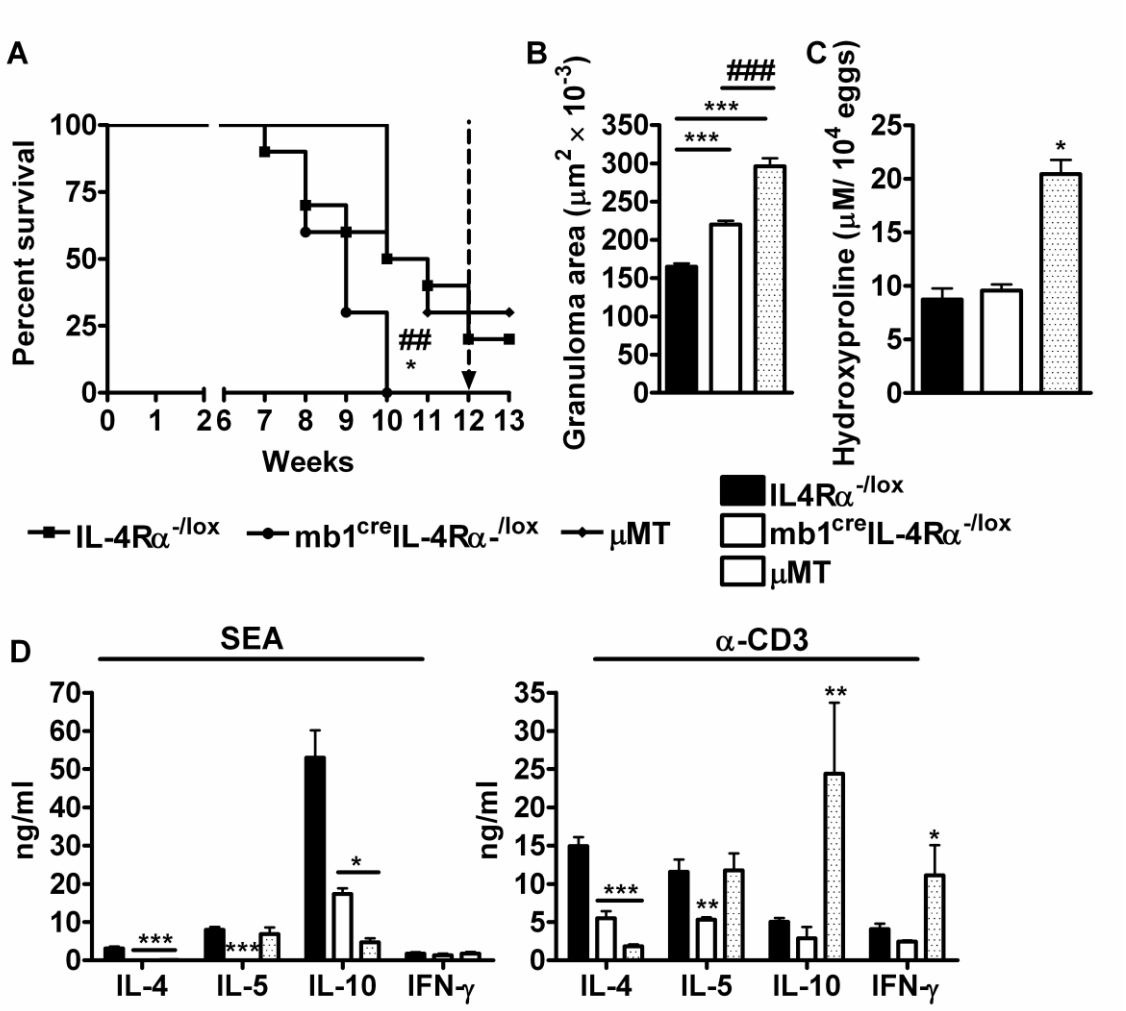

Figure 3.7: IL-4/IL-13 responsive B cells confer protection to the host against *S. mansoni* infection.
IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and μMT mice were infected with 100 *S. mansoni* cercariae and analysed 7 weeks post-infection. (**A**) Survival kinetics of mice infected with *S. mansoni* (n= 8-10 mice). Survival curves were compared using Logrank test. *p< 0.05 and **p< 0.01 vs IL-4Rα$^{-/lox}$ mice.(**B**) Granuloma area measured by microscopic analysis of formalin-fixed liver sections after H&E staining. (**C**) Liver fibrosis measured as hydroxyproline content normalised to tissue egg numbers (mean ± SEM, n= 4-6). (**D**) Cytokine production by total mesenteric lymph node cells re-stimulated with either SEA or α-CD3 (mean ± SEM, n= 4-6). Data are representative of two independent experiments. *p< 0.05, **p< 0.01 and ***p< 0.001 vs IL-4Rα$^{-/lox}$ mice.

B cell-specific IL-4Rα deficient mice fail to down-modulate liver and gut granuloma formation during chronic schistosomiasis

B cells are known to play a crucial role in modulating *S. mansoni* egg-induced granuloma formation during chronic stages of infection [233]. To determine whether IL-4Rα expression on B cells is required to regulate liver pathology during chronic stages of infection, we infected *mb1*^{cre}IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and IL-4Rα$^{-/lox}$ mice with 30 live *S. mansoni* cercariae and killed mice at 16 and 24 weeks post-infection. *Mb1*^{cre}IL-4Rα$^{-/lox}$ mice failed to down-regulate granuloma formation during the chronic stages of infection, displaying granulomas that were 3-fold bigger than those from the littermate control mice (Fig 3.8A). In contrast, IL-4R$^{-/-}$ mice consistently displayed smaller granulomas at both time points compared to littermate control mice (Fig 3.8A).

Analysis of systemic responses revealed that there was no significant difference in the serum levels of IL-4 between the mutant mouse strains at 16 weeks post-infection (Fig 3.8B). However, sera from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice contained elevated levels of IL-4 at 24 weeks post-infection compared to littermate control mice (Fig 3.8B). These data suggest that IL-4/IL-13 responsive B cells are required to down-regulate granuloma formation during the chronic stages of infection.

To investigate the importance of B cell-specific IL-4Rα expression in regulating cellular immunity during chronic stages of infection, MLN cells from chronically infected mice were stimulated with the mitogen α-CD3 and cytokines were detected by ELISA. While IL-10 was significantly reduced in *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice compared to littermate control mice, the levels of IL-4, IL-5 and IFN-γ were similar between the mutant mouse strains at 16 weeks post-infection (Fig 3.8C). In contrast, at 24 weeks post-infection, cells from B cell-specific IL-4Rα deficient mice produced augmented levels of Th1, Th2 and Th17 cytokines compared to littermate control mice, while cells from IL-4Rα$^{-/-}$ mice produced increased levels of Th1 and Th17 cytokines only (Fig 3.8D). Histological examination of H&E stained liver section from mice killed at 16 and 24 weeks post-infection confirmed prior findings, where *mb1*^{cre}IL-4Rα$^{-/lox}$ mice were found to have exacerbated liver granuloma formation compared to littermate control mice (Fig 3.8E). Interestingly, B cell-specific IL-4Rα deficient mice and global IL-4Rα knock-out mice both had increased mortality during the chronic stages of infection compared to littermate control mice (data not shown). Furthermore, there was shunting of *S. mansoni* eggs into the lungs and spleen of *mb1*^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ mice at both 16 and 24 weeks post-infection, whereas no eggs were detected in the lungs and spleen of IL-4Rα$^{-/-}$ mice (data not shown). In addition, *Mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice exhibited enhanced gut inflammation, indicated by increased inflammatory cells surrounding the eggs at both time points, while the IL-4Rα$^{-/lox}$ mice controlled gut inflammation (Fig 3.9A). This suggests that B cell-specific IL-4Rα expression in crucial for down-regulating gut pathology during the chronic stages of *S. mansoni* infection. Together, these data suggest that the absence of IL-4Rα expression on B cells leads to dysregulated immune responses and granuloma formation during the chronic stages of *S. mansoni* infection.

Enhanced lung granuloma formation and fibrosis in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice challenged with synchronous *S. mansoni* eggs

To confirm the importance of IL-4/IL-13 responsive B cells in down-modulating tissue pathology during infection, we utilised a synchronous *S. mansoni* pulmonary model where mice were sensitised for two weeks by intraperitoneal injection of 2 500 *S. mansoni* eggs, subsequently challenged intravenously with 2 500 eggs and then killed at day 7 and 14 post-challenge. *Mb1*^{cre}IL-4Rα$^{-/lox}$ mice had augmented granuloma formation at both time points compared to the littermate control mice (Fig 3.10A). There was no significant difference in lung fibrosis in B cell-specific IL-4Rα deficient mice at day 7 post-challenge but by day 14 fibrosis was significantly increased compared to littermate control mice (Fig 3.10B). Histological examination of H&E

stained lung sections showed that B cell-specific IL-4Rα deficient mice had bigger granulomas than littermate control mice at both time points (Fig 3.10C). Therefore, these data strongly support our initial conclusion that B cell-specific IL-4Rα expression is crucial for down-modulating granuloma formation and fibrosis in mice.

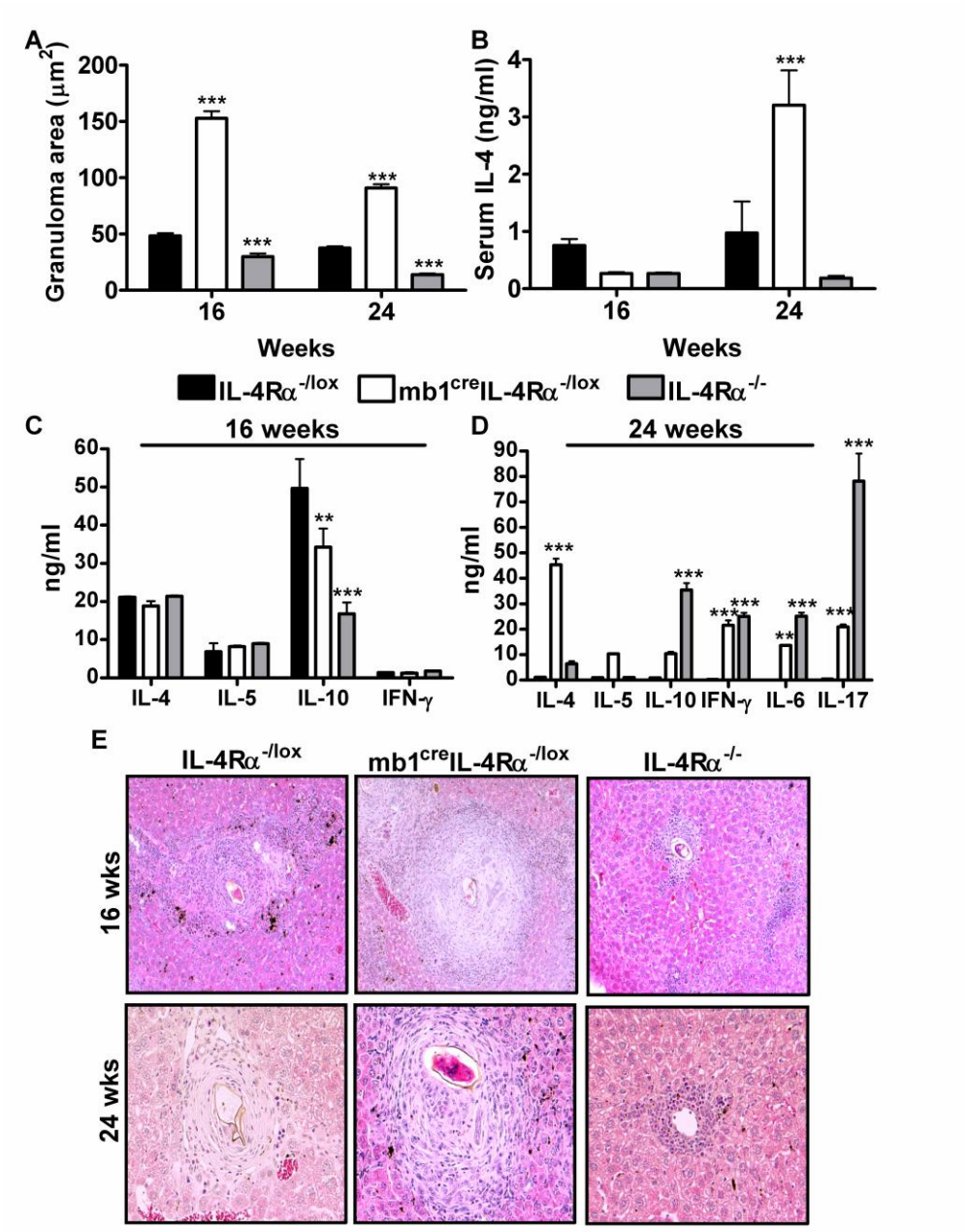

Figure 3.8: IL-4/IL-13 responsive B cells are required for down-regulating hepatic pathology during chronic schistosomiasis.
IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 30 *S. mansoni* cercariae and killed at 16 and 24 weeks post-infection. (**A**) Liver granuloma area was measured using a computerised morpho-metric analysis program (NIS elements by NIKON) by measuring 20-25 granulomas per mouse. (**B**) Serum IL-4 levels were detected by ELISA at both time points. (**C-D**) Detection of cytokine production by MLN cells after in vitro restimulation with α-CD3 for 72 h. (**E**) Histology images showing liver granuloma formation at 16 and 24 weeks post-infection. Data represents two independent experiments. **p<0.01, ***p< 0.001 vs IL-4Rα$^{-/lox}$ mice. n = 4-6 mice per group.

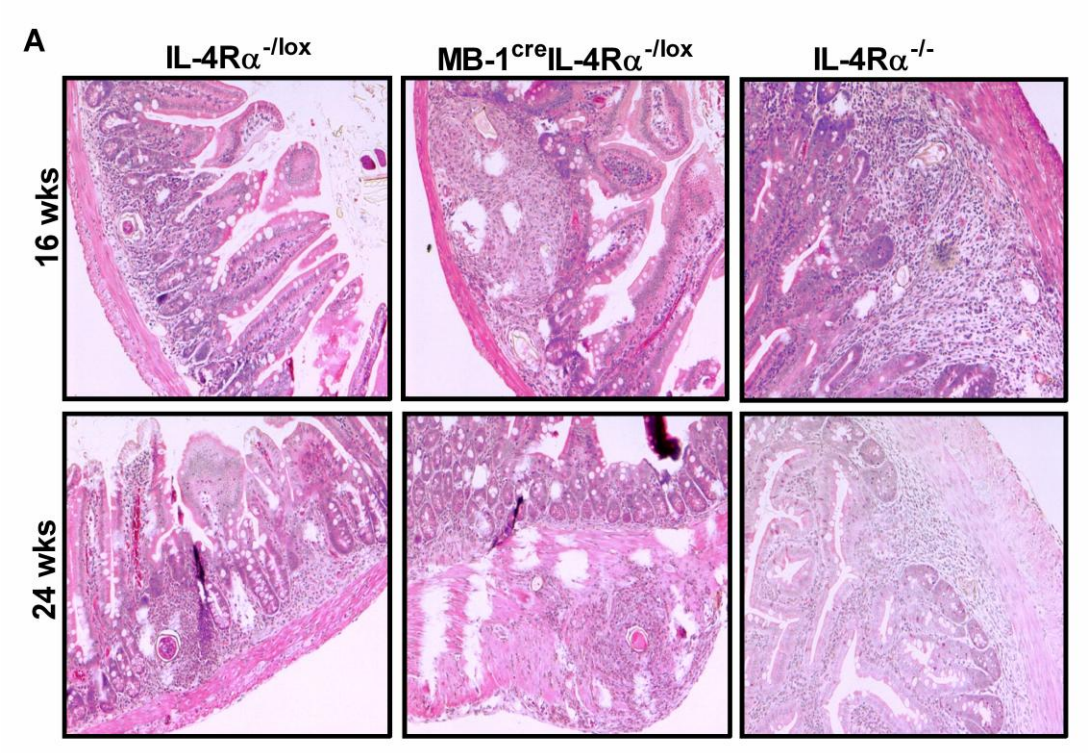

Figure 3.9: Augmented gut inflammation in B cell-specific IL-4Rα deficient mice during chronic schistosomiasis.
IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 30 *S. mansoni* cercariae and analysed at 16 and 24 weeks post-infection. (**A**) Histological examination of gut tissue after staining sections with H&E (×100). Data represents two independent experiments. n= 4-6 mice per group.

We next examined the cellular immune responses to *S.mansoni* eggs in B cell-specific IL-4Rα deficient mice. Here, mediastinal lymph node (MST) cells from challenged mice were restimulated with α-CD3 *ex vivo* and cytokines were detected by ELISA. There was no significant difference in the production of Th1 and Th2 cytokines by cells from both mutant mouse strains that received mitogenic stimulation after 7 days post-challenge (Fig 3.11A). However at day 14 post-challenge, cells from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice responded to mitogenic stimulation by up-regulating the production of IL-4, IL-10 and IFN-γ compared to littermate control mice (Fig 3.11B). Furthermore, mice deficient in IL-4Rα expression on B cells had significantly reduced absolute numbers of CD3^{+}CD4^{+} T cells and CXCR5^{+} T_{FH} cells (Fig 3.11C & D). Although the number of effector CD4^{+} T cells was not altered at day 7 post-challenge between the mutant mouse strains, mice lacking IL-4Rα expression on B cells had diminished numbers of effector CD4^{+} T cells at day 14 post-challenge compared to littermate control mice (Fig 3.11E). Finally, abrogation of IL-4Rα expression on B cells significantly impaired intracellular cytokine production by CD3^{+}CD4^{+} T cells at both time points, indicated by reduced number of IL-4, IL-10 and IFN-γ secreting cells after stimulation with PMA/Ionomycin compared to littermate control mice. Therefore, these data suggest that IL-4Rα expressing B cells influence CD4^{+} T cell cellular responses during *S. mansoni* eggs challenge.

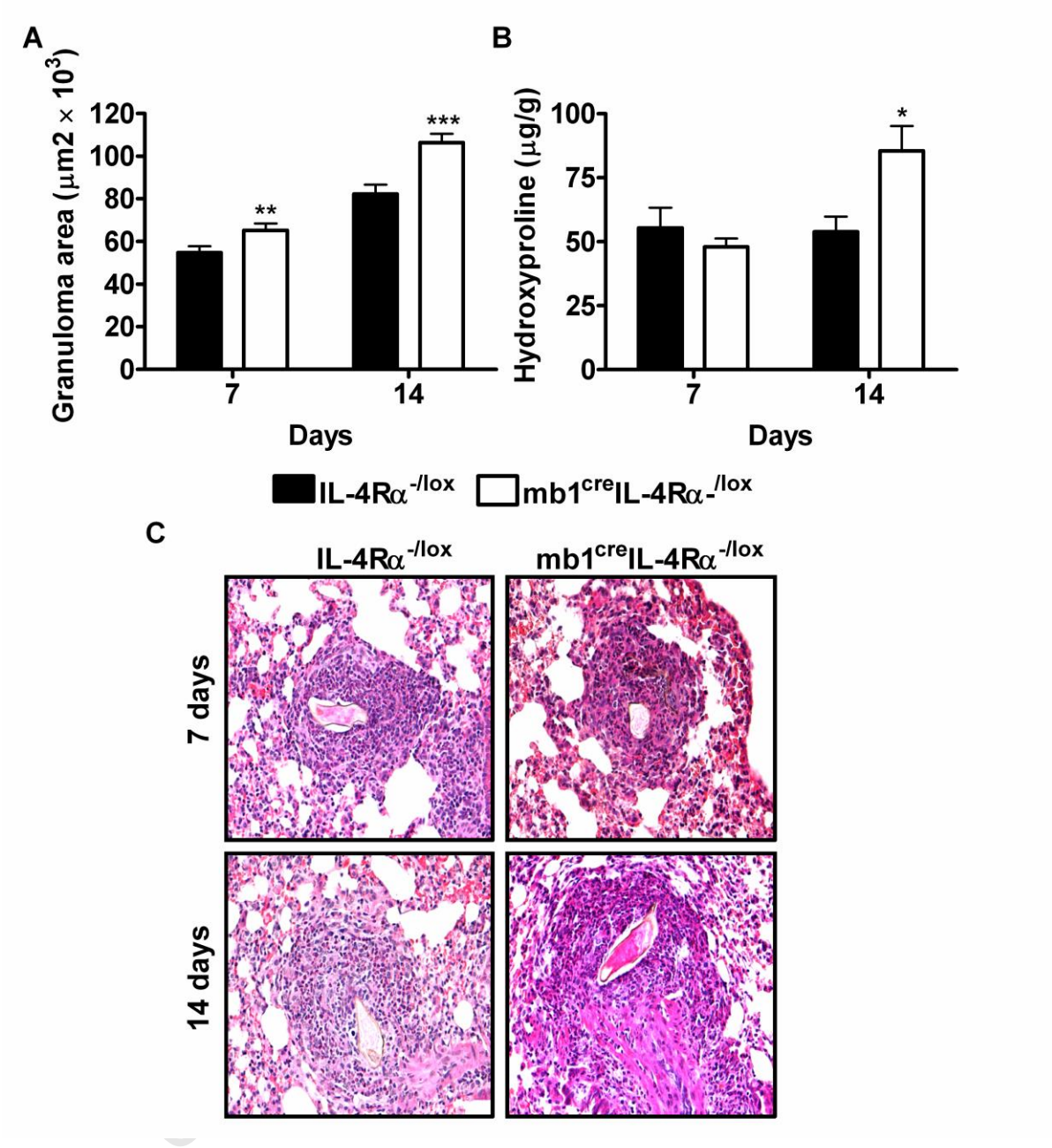

Figure 3.10: Exacerbated lung granuloma formation and fibrosis in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice challenged intravenously with *S. mansoni* eggs.

IL-4Rα$^{-/lox}$, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were sensitised with 2 500 S. mansoni eggs intraperitoneally, challenged with 2 500 eggs intravenously 14 days later and killed over two time points (7 and 14 days post-challenge). (**A**) Granuloma formation measured using a computerised morpho-metric analysis program (NIS elements by NIKON) by measuring 20-25 granulomas per mouse. (**B**) Lung fibrosis measured by determining hydroxyproline concentration. (**C**) Histological examination of H&E stained lungs sections. Data represents two independent experiments. *p< 0.05, **p< 0.01 and ***p< 0.001 vs IL-4Rα$^{-/lox}$ mice. n= 6 mice per group.

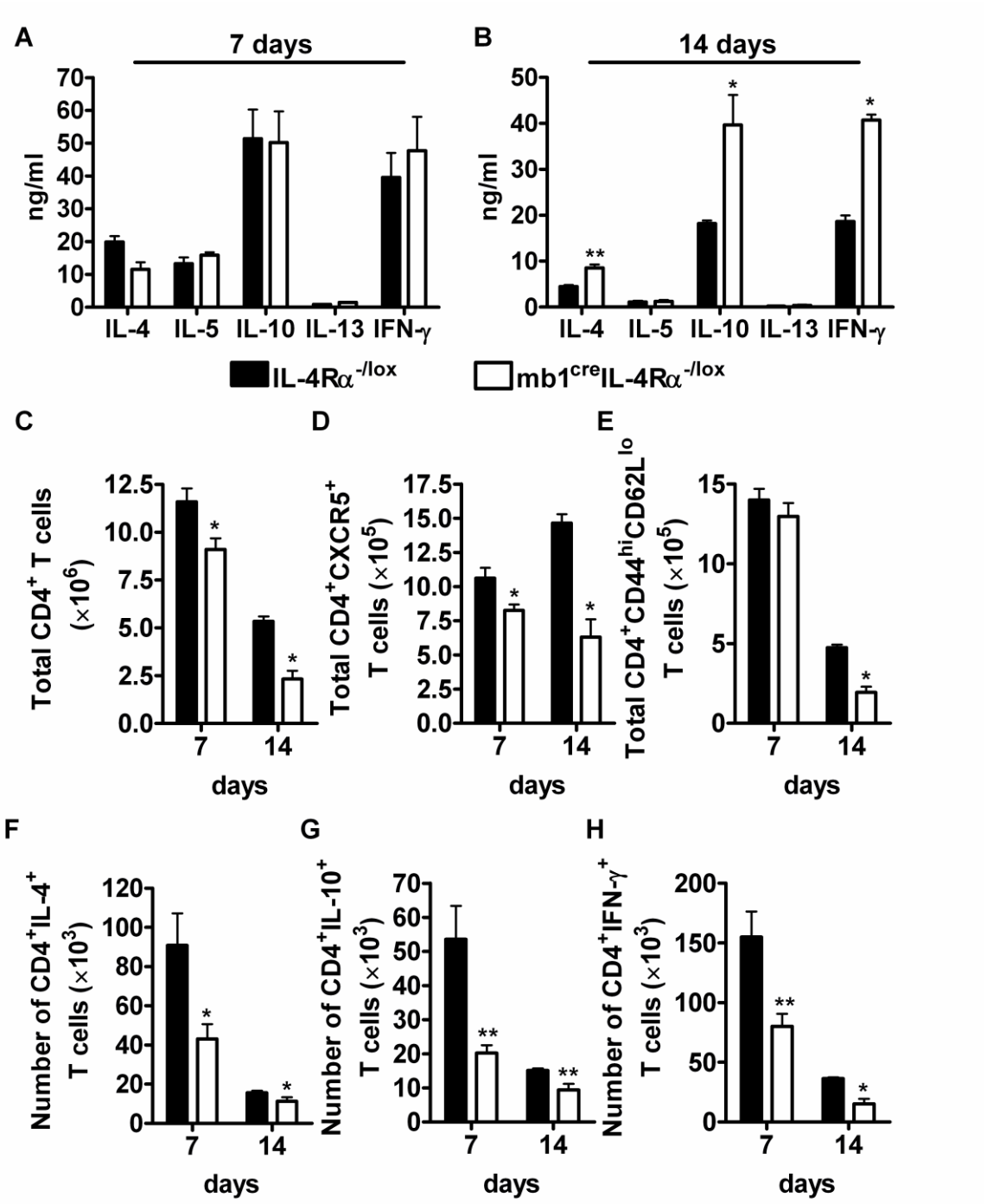

Figure 3.11: Impaired recruitment of $CD4^+$ T cells, T_{FH} cells and effector $CD4^+$ T cells in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice challenged with *S. mansoni* eggs.

Single cell suspensions were prepared from mediastinal lymph nodes (MST) and cells were stained for flow cytometry. (**A-B**) Cytokine production by total MST cells restimulated with α-CD3 *in vitro*, detected by ELISA. (**C-E**) Recruitment of $CD3^+CD4^+$ T cells, $CXCR5^+$ T_{FH} cells and effector $CD4^+$ T cells into the lung draining lymph nodes. (**F-H**) Intracellular cytokine secretion by $CD4^+$ T cells after restimulation of total MST cells with 50 ng/ml PMA and 250 ng/ml ionomycin. Data are representative of two independent experiments. *p< 0.05, **p<0.01 vs IL-4Rα$^{-/lox}$ mice. n= 6 mice per group.

Impaired B cells responses in mice deficient in IL-4Rα expression on B cells during *S. mansoni* egg-challenge

To investigate the requirement for IL-4Rα expression on B cells during differentiation and cytokine production by B cells, we prepared single cell suspensions from MST and stained the cells for flow cytometric analysis. The numbers of $CD19^+B220^+$ B cells (Fig 3.12A) and follicular B cells (Fig 3.12B) were not altered between the mutant mouse strains at day 7 post-challenge, however, at day 14 post challenge *mb1*^{cre}IL-4Rα$^{-/lox}$ mice had reduced total numbers of $CD19^+B220^+$ B cells (Fig 3.12A) and follicular B cells (Fig 3.12B) compared to IL-4Rα$^{-/lox}$ mice. The total number of marginal zone B cells was enhanced in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice at day 7 post-challenge but remained the same at day 14 post-challenge in comparison to littermate control mice (Fig 3.12C). There was a general impairment of intracellular cytokine secretion by $CD19^+$ B cells from B cell-specific IL-4Rα deficient mice at both time points compared to littermate control mice (Fig 3.12D, E & F). These data suggest that IL-4Rα expression on B cells is required for differentiation of B cell subsets and optimal secretion of cytokines.

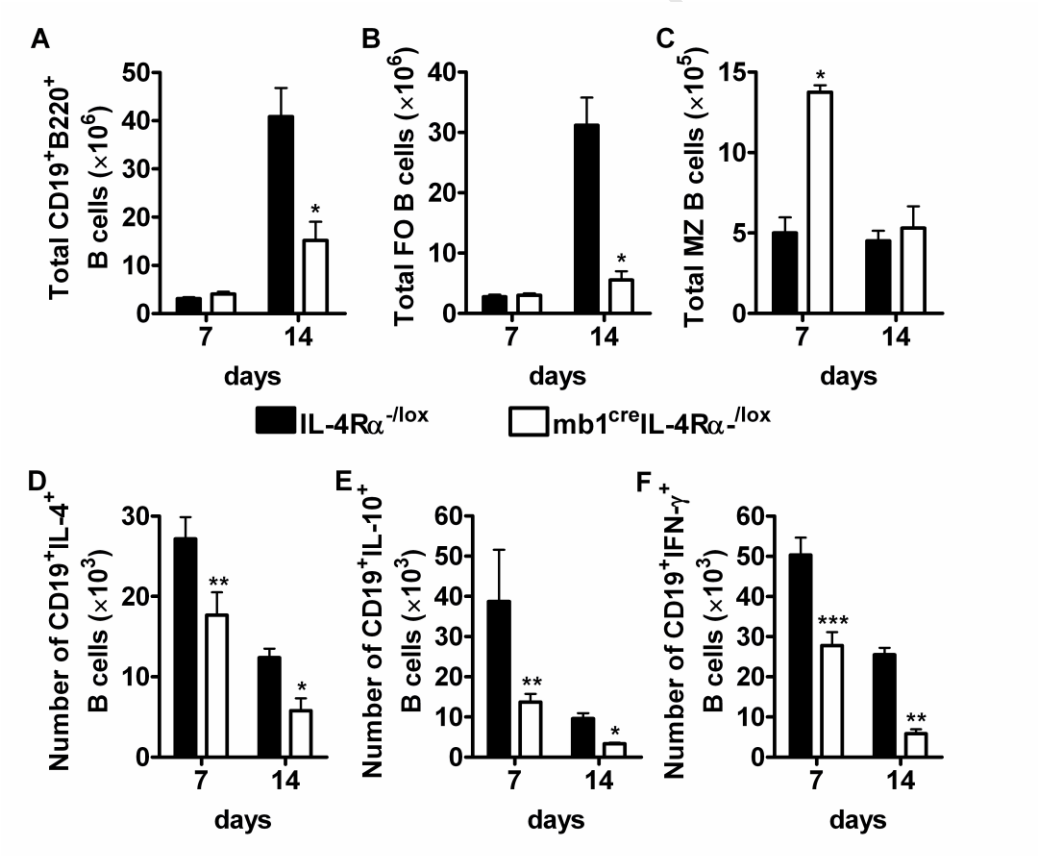

Figure 3.12: Impaired recruitment of $CD19^+B220^+$ B cells in B cell-specific IL-4Rα deficient mice challenged with *S. mansoni* eggs.
Single cell suspensions were prepared from MST and cells were stained for flow cytometry. (**A-C**) Recruitment of $CD19^+B220^+$ B cells, FO B cells and MZ B cells into the MST. (**D**) Intracellular cytokine detection after stimulating total MST cells with 50 ng/ml PMA and 250 ng/ml ionomycin *in vitro*. Data represents two independent experiments. *p< 0.05, **p<0.01, ***p<0.001 vs IL-4Rα$^{-/lox}$ mice.

Macrophages displayed a classically activated profile in the absence of IL-4Rα expression on B cells

To determine the activation status of macrophages during *S. mansoni* egg-challenge in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice, we prepared single cell suspensions from MST cells and stained for flow cytometric analysis. At day 7 post-challenge, there was no significant difference in the expression of the alternative activation markers arginase 1 (Fig 3.13A) and Ym-1 (Fig 3.13B), as well as the classical activation marker iNOS (Fig 13C) in CD11b^{+}MHCII^{+} cells from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice. However, CD11b^{+}MHCII^{+} macrophages from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice exhibited a shift towards classical activation at day 14 post-challenge by up-regulating the expression of iNOS with a concomitant down-regulation of Arginase and Ym-1 compared to littermate control mice (Fig 3.13A, B & C). Recruitment of Siglec-F^{+} eosinophils into the draining lymph node was unaltered at day 7 post-challenge but their number was significantly reduced at day 14 post-challenge in B cell-specific IL-4Rα deficient mice compared to the littermate control mice (Fig 3.13 D). These data may either suggest that a cytokine microenvironment in the draining lymph nodes of *mb1*^{cre}IL-4Rα$^{-/lox}$ mice influences the macrophage activation profile or that B cells expressing IL-4Rα act directly on macrophages, thus, influencing their activation status.

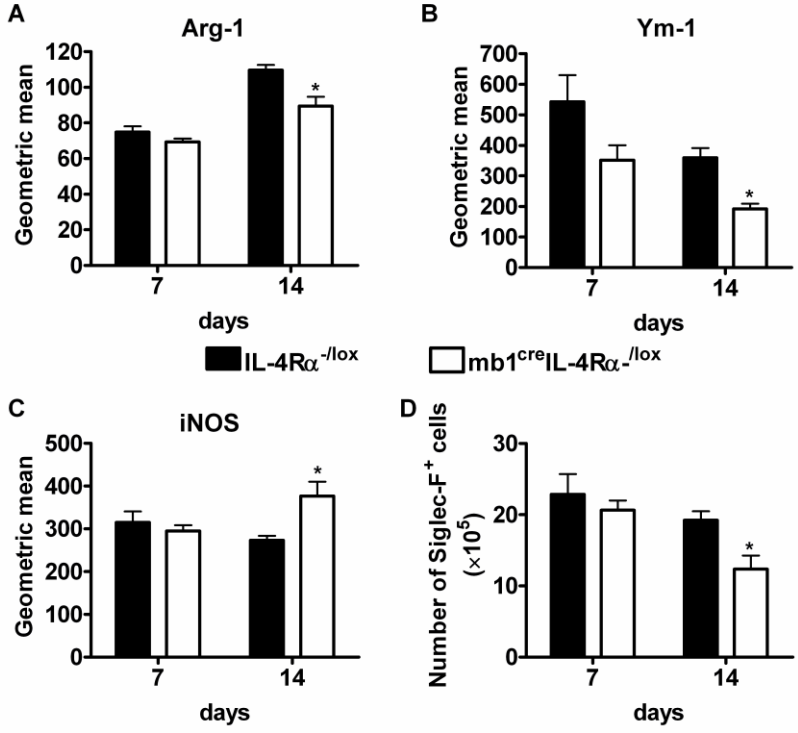

Figure 3.13: Increased expression of iNOS by alveolar macrophages from B cell-specific IL-4Rα deficient mice challenged with *S. mansoni* eggs.
Single cell suspension was prepared from lungs and cells were stained for flow cytometric analysis. (**A**-**B**) Expression of Arg-1 and Ym-1 by alveolar macrophages. (**C**) Expression of iNOS by alveolar macrophages. (**D**) Absolute number of Siglec-F^{+} eosinophils draining into the lymph node. Data represents two independent experiments. **p< 0.05 vs IL-4Rα$^{-/lox}$ mice. n= 6 mice per group.

Generation of chimeric mice lacking IL-4 production by B cells

We have successfully established that IL-4Rα expression on B cells is essential in the development of host protective immunity against *S. mansoni* infection. However, we have not determined the cellular source of the IL-4 that mediates IL-4Rα signalling on B cells leading to protective immunity against infection. Previous studies have demonstrated the capacity of B cells to produce IL-4 during *Hp* infection [106]. Since there is no transgenic mouse strain that has impaired production of IL-4 specifically on B cells, we decided to exploit the mixed bone marrow chimera method because it allowed us to selectively express or delete a particular gene in a specific hematopoietic lineage cell [90,277]. Here, lethally irradiated B cell-deficient mice (μMT) were reconstituted with 80% μMT and 20% IL-4$^{-/-}$ bone marrow (BM) to generate chimera that lacked IL-4 expression specifically on B cells (B-IL-4$^{-/-}$). As controls, we reconstituted recipient mice with a mixture of 80% μMT and 20% Balb/c BM to generate wild-type chimera (WT), sufficient in IL-4 expression in all hematopoietic cells. Finally, to generate chimera that have impaired IL-4 expression in all hematopoietic cells we reconstituted recipient mice with 100% IL-4$^{-/-}$ BM (IL-4$^{-/-}$). All the chimeric mice contained equivalent proportions of CD3^{+}CD4^{+} T cells and CD19^{+}B220^{+} B cells in peripheral blood 8 weeks after reconstitution, indicating successful reconstitution (Fig 3.14).

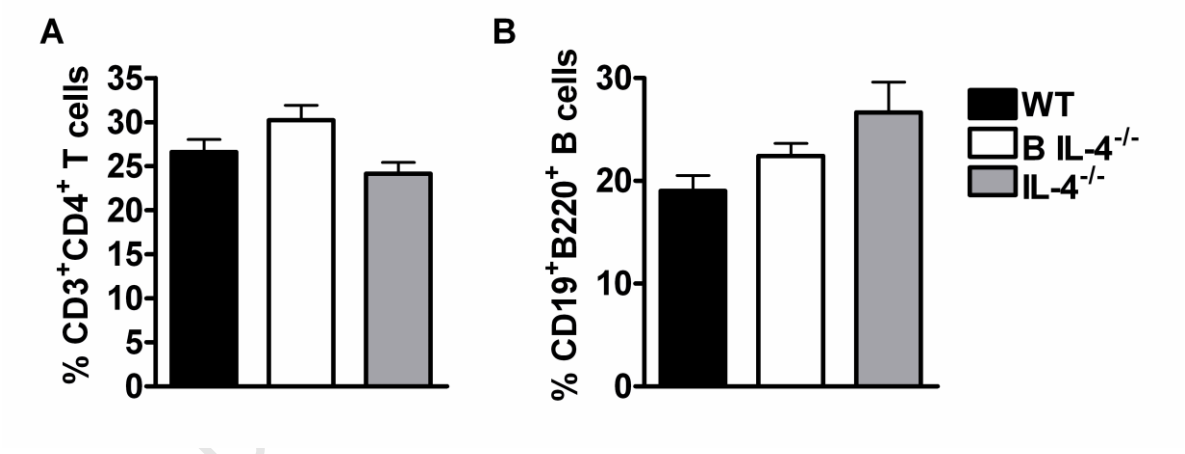

Figure 3.14: Efficient bone marrow reconstitution of irradiated recipient μMT mice.
Irradiated μMT mice were reconstituted with 80% μMT and 20% Balb/c BM (WT), 80% μMT and 20% IL-4$^{-/-}$ BM (B IL-4$^{-/-}$) or 100% IL-4$^{-/-}$ BM (IL-4$^{-/-}$) and allowed to reconstitute for 8 weeks. Mice were bled at 8 weeks and cells were stained for flow cytometric analysis. (**A**) Proportions of CD3^{+}CD4^{+} T cells found in blood after reconstitution. (**B**) Proportions of CD19^{+}B220^{+} B cells found in blood after reconstitution. Data represent two independent experiments. n= 6 mice per group.

To further confirm successful generation of the mixed bone marrow chimeric mice, we infected chimeras with 100 live *S. mansoni* cercariae and collected sera at 7 week post-infection by cardiac puncture. As expected, WT and B-IL-4$^{-/-}$ chimeras made SEA-specific IgG1 and total IgE antibodies in response to *S. mansoni* infection (Fig 3.15A & B). Concomitantly, WT and B-IL-4$^{-/-}$ chimeras failed to produce antigen specific type 1 (IgG2a and IgG2b) antibody isotypes (Fig 3.15C & D). In contrast, infected IL-4$^{-/-}$ chimeras failed to class switch antibody isotypes as demonstrated by a robust SEA-specific IgG2a and IgG2b type 1 response (Fig 3.15C & D) and a

diminished type 2 antibody response (Fig 3.15A & B). These data demonstrate that sufficient antibody responses develop in the absence of B cell-specific IL-4 production.

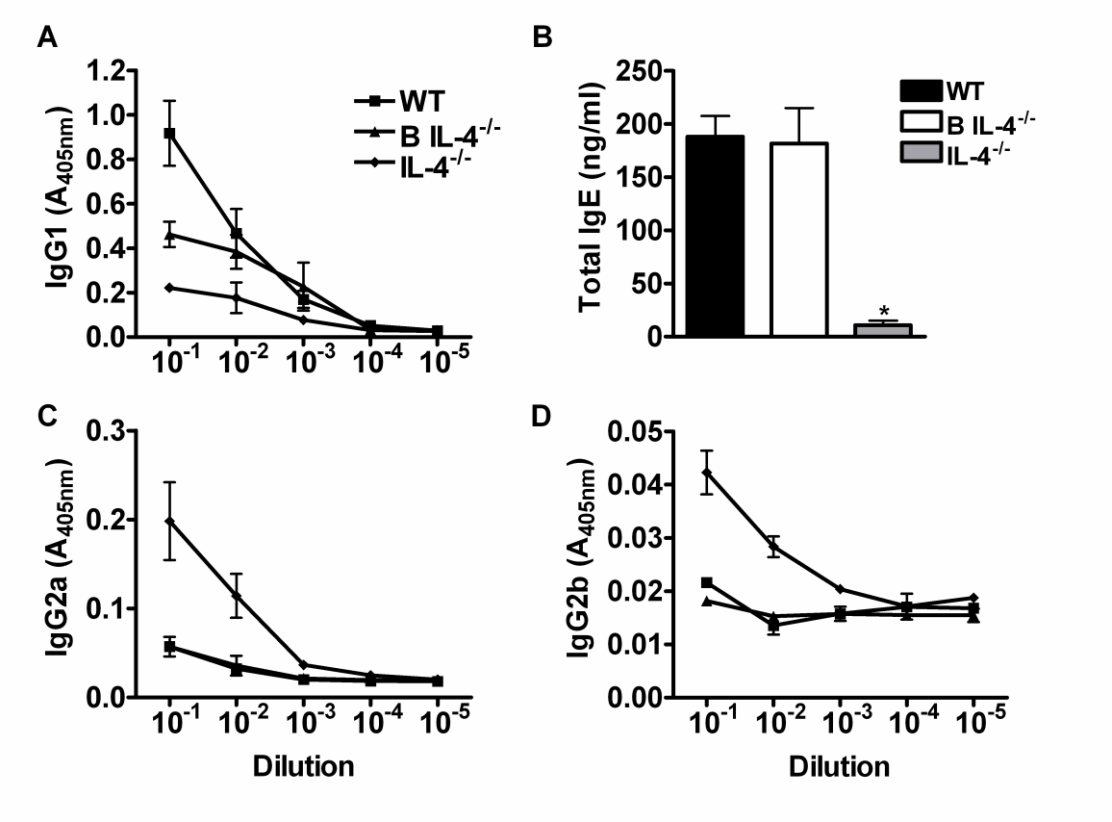

Figure 3.15: Sufficient humoral immunity develops in mice lacking IL-4 producing B cells during *S. mansoni* infection.
Irradiated µMT mice were reconstituted with 80% µMT and 20% Balb/c BM (WT), 80% µMT and 20% IL-4$^{-/-}$ BM (B IL-4$^{-/-}$) or 100% IL-4$^{-/-}$ BM (IL-4$^{-/-}$) and infected with 100 *S. mansoni* cercariae. Mice were killed 7 weeks post-infection and blood was collected for serum preparation. (**A-D**) Serum antibody titers detected by ELISA. Data represent two independent experiments. *p<0.05 vs WT mice. n= 6 mice per group.

Reduced granuloma formation in B cell-specific IL-4 deficient mice infected with *S. mansoni*

To investigate whether IL-4 expressing B cells contribute towards limiting tissue pathology during *S. mansoni* infection, we measured liver granuloma size from H&E stained liver sections from infected chimeric mice. B-IL-4$^{-/-}$ chimeras had significantly reduced granuloma size (Fig 3.16A) but similar tissue fibrosis (Fig 3.16B) and levels of serum AST, an indicator of hepatocellular damage (Fig 3.16C) compared to WT control mice. As expected, IL-4$^{-/-}$ chimeras had significantly augmented granuloma size while hydroxyproline concentration remained similar to WT control chimeras (Fig 3.16A & B). However, IL-4$^{-/-}$ chimeras had increased serum AST levels than WT chimeras, indicating increased hepatocellular damage (Fig 3.16 C). Examination of H&E stained liver sections confirmed that B-IL-4$^{-/-}$ chimeras develop smaller granulomas than WT chimera while IL-4$^{-/-}$ chimera have bigger granulomas (Fig 3.16 D). Interestingly, iNOS positive cells surrounded the granuloma egg in IL-4$^{-/-}$ chimeras while iNOS positive cells were

not detected in granulomas from infected B-IL-4$^{-/-}$ and WT chimeras (Fig 3.16D). Together, these data demonstrate that IL-4 producing B cells are not involved in limiting tissue destruction during *S. mansoni* infection.

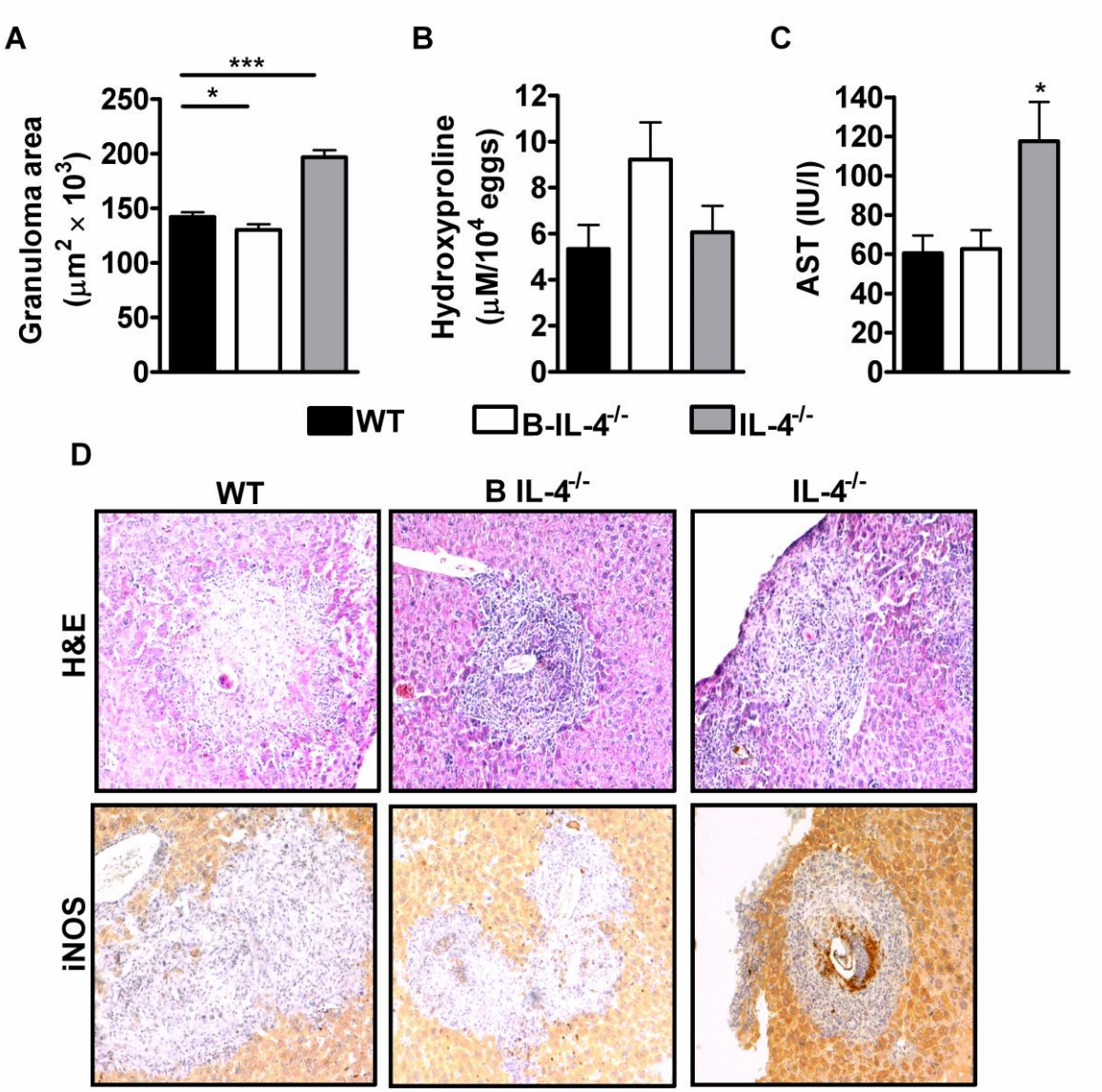

Figure 3.16: Reduced granuloma formation and unaltered liver fibrosis in B IL-4$^{-/-}$ mice.
Irradiated µMT mice were reconstituted with 80% µMT and 20% Balb/c BM (WT), 80% µMT and 20% IL-4$^{-/-}$ BM (B IL-4$^{-/-}$) or 100% IL-4$^{-/-}$ BM (IL-4$^{-/-}$) and infected with 100 *S. mansoni* cercariae. (A) Granuloma area was measured using computerised morph-metric analysis (NIS Elements, Nikon) by measuring 20-25 granulomas per mouse. (B) Liver fibrosis determined by assaying hydroxyproline concentration normalised to tissue eggs. (C) Hepatocellular damage indicated by serum AST concentration. (D) Histological examination of H&E and iNOS stained liver sections. Data are representative of two independent experiments. *p<0.05, ***<0.001 vs WT mice. n= 6 mice per group.

Unaltered development of Th2 immunity and differentiation of $CD4^+$ T cells in B-IL-$4^{-/-}$ chimera mice

IL-4 producing B cells play a crucial role during the differentiation of Th2 cells *in vitro* [106]. To test whether IL-4 produced by B cells is required for the generation of Th2 immune responses during *S.mansoni* infection, we cultured MLN cells from infected chimeric mice with either SEA or α-CD3 for 72 hours and detected cytokine production by ELISA. There was no significant difference in the production of both Th1 and Th2 cytokines by infected B-IL-$4^{-/-}$ and WT chimeras after restimulation with SEA *ex vivo* (Fig 3.17A). However, cells from infected IL-$4^{-/-}$ chimeras failed to produce IL-4, IL-6, IL-10 and IL-13 in response to antigen-specific stimulation *ex vivo* compared to WT control mice (Fig 3.17A). Mitogenic restimulation of cells from infected B-IL-$4^{-/-}$ chimeras resulted in similar production of IL-4, IL-5, IL-10 and IFN-γ but significantly reduced IL-6 and IL-13 compared to WT chimeras (Fig 3.17B). As expected, IL-4 was not detected in supernatant from infected IL-$4^{-/-}$ chimera cells stimulated with the mitogen and other Th2 cytokines were similarly reduced (Fig 3.17B). Therefore, these data suggest that B cell-specific IL-4 production is not required for generation of Th2 cytokine responses during *S. mansoni* infection.

To investigate whether IL-4 produced by B cells contributes to the differentiation and activation of $CD4^+$ T cells in the draining lymph nodes, we prepared single cell suspensions from MLN of infected chimeras and stained the cells for flow cytometric analysis. We found no significant difference in the absolute number of $CD3^+CD4^+$ T cells (Fig 3.17C), $CD4^+$ T cells expressing ICOS (Fig 17D) and effector $CD4^+$ T cells expressing $CD44^{hi}CD62L^{lo}$ (Fig 3.17E) between B-IL-$4^{-/-}$ and WT chimeras. In contrast, although there was no significant difference in the number of $CD3^+CD4^+$ T cells between IL-$4^{-/-}$ and WT chimeras, differentiation of ICOS expressing $CD4^+$ T cells (Fig 3.17D) and expansion of effector $CD4^+$ T cells were impaired in IL-$4^{-/-}$ chimeras (Fig 3.17E). These data demonstrate that the absence of IL-4 production by all hematopoietic cells results in impaired differentiation of $CD4^+$ T cells during infection.

Differentiation of B cells is not altered in chimeric mice deficient in B cell IL-4 production

To explore the impact of abrogation of IL-4 production by B cells on their activation profile and cytokine production, we prepared cells from infected chimeric mice and stained for flow cytometric analysis. The absence of IL-4 produced by B cells did not prevent the differentiation of $CD19^+B220^+$ B cells in B-IL-$4^{-/-}$ chimeras compared to WT chimeras (Fig 3.18A). In contrast, differentiation of $CD19^+B220^+$ B cells was impaired in IL-$4^{-/-}$ chimeras compared to WT chimeras, indicating the importance of IL-4 in the differentiation of B cells during infection (Fig 3.18A). Likewise, the expansion of follicular B cells was impaired in IL-$4^{-/-}$ chimeras while there was no significant difference in the absolute number of follicular B cells between B-IL-$4^{-/-}$ and WT chimeras (Fig 3.18B). Numbers of marginal zone B cells were significantly increased in both B-IL-$4^{-/-}$ and IL-$4^{-/-}$ chimeras, with IL-$4^{-/-}$ chimera displaying almost five times more marginal

zone B cells than WT chimeras (Fig 3.18C). Taken together, these data show that differentiation of B cells occurs in the absence of IL-4 production by B cells.

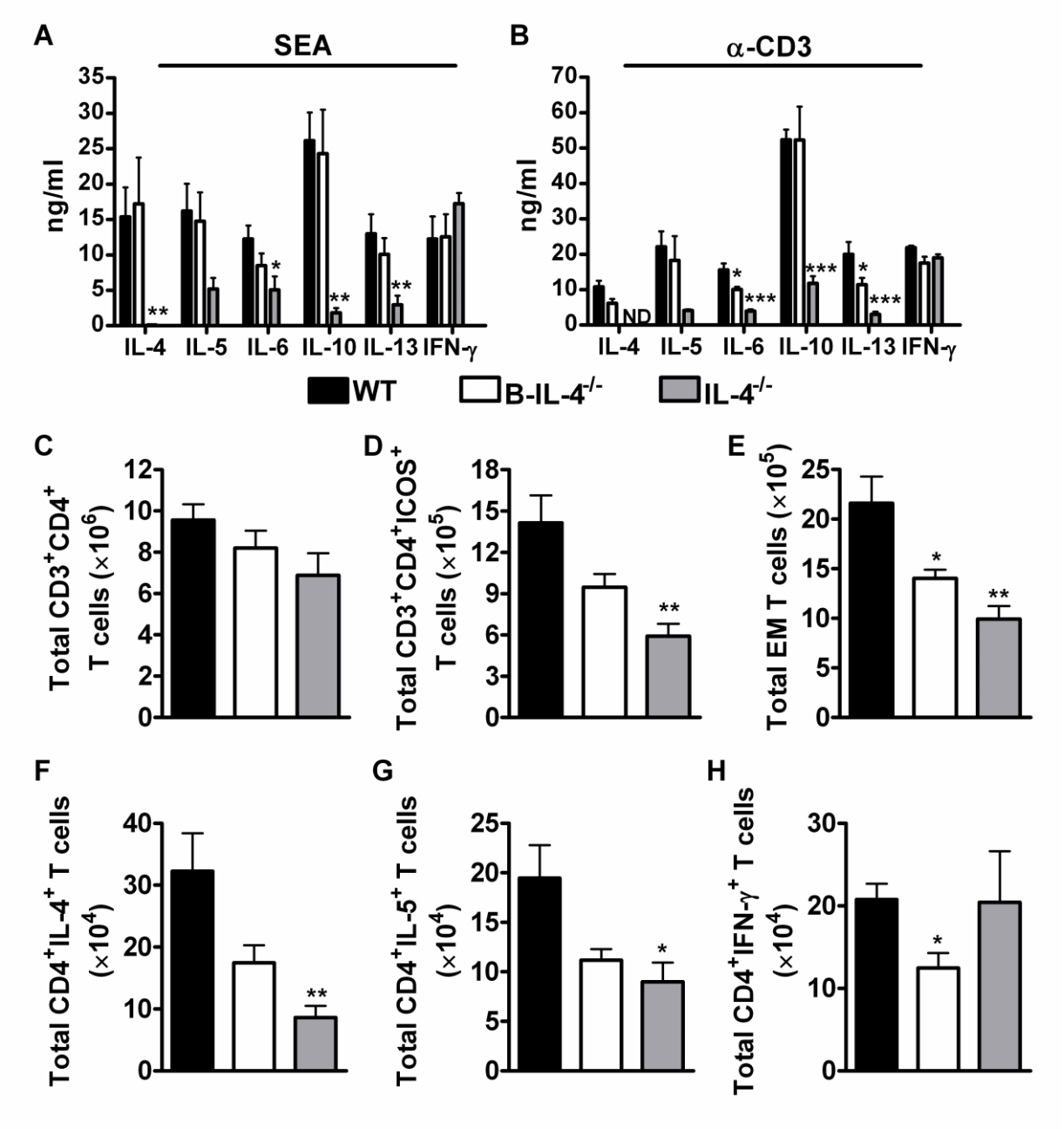

Figure 3.17: Normal Th2 immunity and CD4$^+$ T cell activation in mice deficient of B cell-derived IL-4.
Bone marrow chimera mice were infected with 100 live *S. mansoni* cercariae and killed 7 weeks post-infection. Single cell suspensions were prepared from MLN and cells were restimulated with 20 μg/ml SEA or α-CD3 *ex vivo*. (**A-B**) Cytokine production by restimulated total MLN cells was detected by ELISA. (**C-E**) Recruitment of CD3$^+$CD4$^+$ T cells, ICOS$^+$ T cells and effector CD4$^+$ T cells into draining lymph node. (**F-H**) Intracellular secretion by CD4$^+$ T cells after restimulation of total MLN cells with 50 ng/ml PMA and 250 ng/ml ionomycin. Data are representative of two independent experiments. n= 6 mice per group.

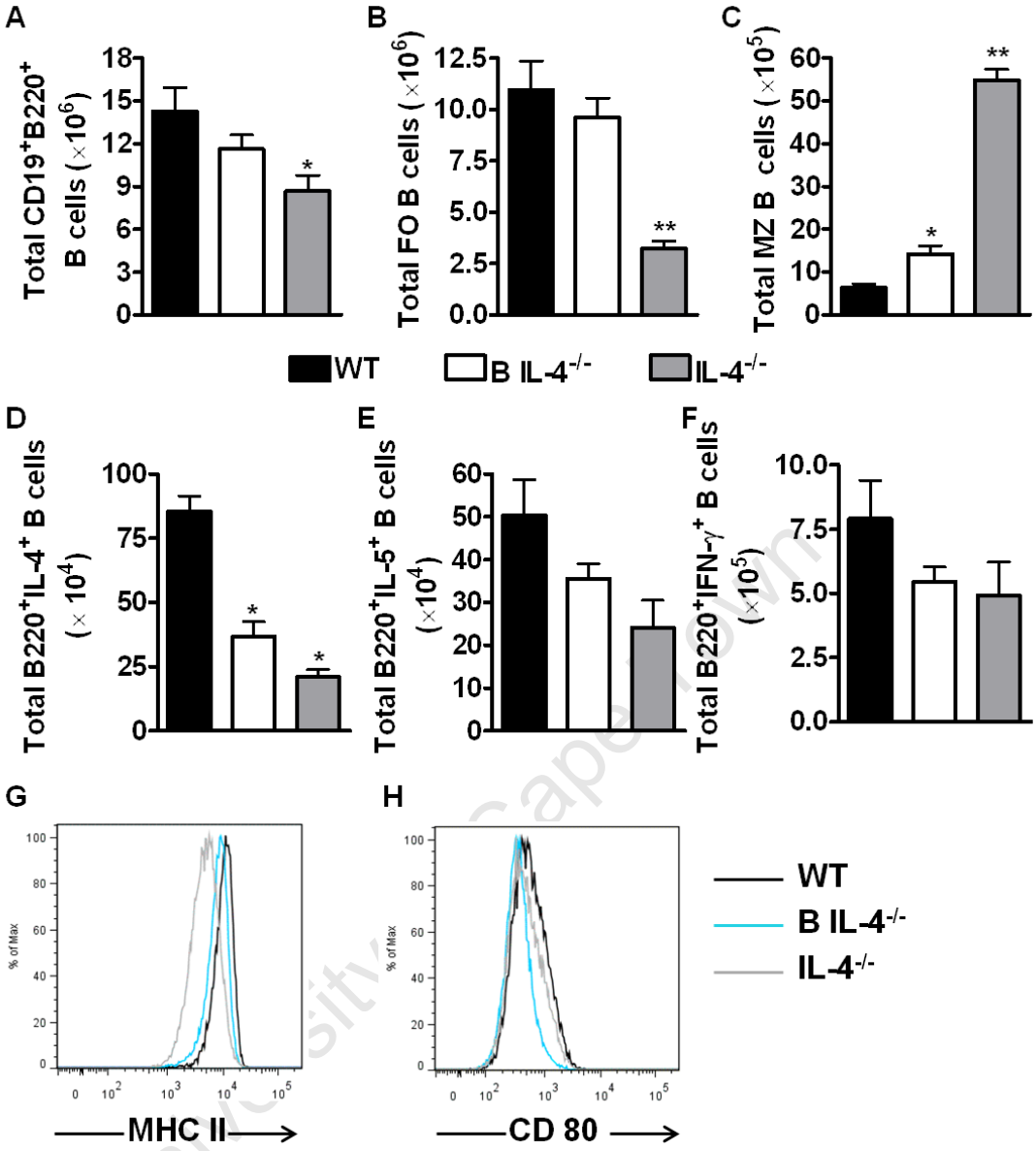

Figure 3.18: Normal recruitment of B cells in mice deficient in IL-4 producing B cells
Single cell suspensions were prepared from MLN of infected bone marrow chimera mice and cells were stained for flow cytometry. (A-C) Recruitment of $CD19^{+}B220^{+}$ B cells, FO B cells and MZ cells into the draining lymph node. (D-F) Intracellular cytokine production by $B220^{+}$ B cells after stimulation of total MLN cells with 50 ng/ml PMA and 250 ng/ml ionomycin. (G-H) Histogram showing the expression of activation markers MHC II and CD80 by $CD19^{+}B220^{+}$ B cells. Data represents two independent experiments. *p<0.05, **p<0.01 vs WT mice. n= 6 mice per group.

Next, to investigate whether the lack of IL-4 production by B cells altered their capacity to produce other cytokines, we analysed intracellular cytokine production by $B220^{+}$ B cells after stimulation of MLN cells with PMA and Ionomycin *ex vivo* and staining for flow cytometric analysis. As expected, intracellular secretion of IL-4 by $B220^{+}$ B cells from B-IL-$4^{-/-}$ and IL-$4^{-/-}$ chimeras was reduced, relative to WT chimeras (Fig 3.18D). However, intracellular production of IL-5 and IFN-γ was not significantly reduced in both B-IL-$4^{-/-}$ and IL-$4^{-/-}$ compared to WT chimeras (Fig 3.18E & F). Finally, we analysed expression of activation markers MHC II and

CD80 on B cells from cells of infected chimeras by flow cytometry. Expression of MHC II and CD80 was impaired on B cells from infected B IL-4$^{-/-}$ and IL-4$^{-/-}$ compared to WT chimeras (Fig 3.18G & H). Therefore, these data suggest that B cells maintain production of both IL-5 and IFN-γ even after the interference with the production of IL-4.

3.4 Discussion

In this study, we examined the role of IL-4/IL-13 responsive B cells during host immune responses to acute and chronic schistosomiasis. We found that B cell-specific IL-4Rα deficient mice are highly susceptible to acute schistosomiasis and this is related to augmented liver pathology, severe gut inflammation, impaired Th2 immunity and abrogated humoral responses. Furthermore, mice deficient in IL-4Rα expression on B cells presented with significantly bigger hepatic and intestinal granulomas than littermate control mice during the chronic stages of infection, indicating the importance of IL-4/IL-13 responsive B cells in down-regulating tissue pathology. Lastly, mice lacking IL-4 expression specifically in B cells developed normal antibody responses, sufficient Th2 immunity and smaller liver granulomas, demonstrating that IL-4 producing B cells are not required to mediate protective immunity to *S. mansoni* infection. Thus, it appears that the ability of B cells to respond to IL-4/IL-13 stimuli is critical for protective immunity against *S. mansoni* infection.

Mb1^{cre}IL-4Rα$^{-/lox}$ mice rapidly succumbed to acute Schistosomiasis and exhibited 100% mortality by 10 weeks post-infection, whereas 40% of littermate control mice were still alive at this time point. Consistent with previous studies, IL-4Rα$^{-/-}$ were found to be highly susceptible to *S. mansoni* infection and had all died by 8 weeks post-infection. IL-4Rα$^{-/-}$ mice displayed severe weight loss that began at the onset of egg-laying and mice had lost almost 20% of their body weight at the time of death. However, there was no difference in the kinetics of weight loss between *mb1*^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ mice until 8 weeks post-infection, when littermate control mice began to recover and gain some weight. Previous studies have found that IL-4Rα$^{-/-}$ mice suffer from multiple organ defects, indicated by severe atrophy of the spleen and liver during the acute stages of infection [147]. Our findings were consistent with previous data since the weight of liver and spleen from IL-4Rα$^{-/-}$ mice was significantly reduced compared to littermate control mice. In contrast, there was no difference in spleen and liver weight between B cell-specific IL-4Rα deficient mice and littermate control mice, where both mouse strains had severe splenomegaly. Mice that quickly succumb to *S. mansoni* infection display two key morbidity factors: hepatocellular damage caused by dysregulated nitric oxide production and endotoxemia caused by destruction of gut integrity [219]. Global IL-4Rα knock-out mice displayed severe haemorrhaging of the gut indicated by severe bleeding and bloody stool. Similarly, *mb1*^{cre}IL-4Rα$^{-/lox}$ mice had slight haemorrhaging in the gut accompanied by colitis-type pathology whereas littermate control mice maintained gut integrity. Therefore, the inability of B cells to respond to IL-4 signalling is detrimental to host survival during acute schistosomiasis than their ability to produce this cytokine.

Development of a polarised Th2 immune response is indispensable for host survival during *S. mansoni* infection [218,222]. Interestingly, mice deficient in IL-4Rα expression on B cells displayed abrogated antigen-specific Th2 immunity, indicated by impaired production of IL-4 and IL-5 compared to littermate control mice. As expected, IL-4Rα$^{-/-}$ mice displayed a shift towards Th1 immune responses that was accompanied by significant production of IFN-γ after antigen specific restimulation of MLN cells and concomitant reduced production of Th2 cytokines. Therefore, it is plausible that the impaired Th2 immune response contributes to the mechanism underlying susceptibility to acute schistosomiasis in B cell-specific IL-4Rα deficient mice. In a study by Hoffman and colleagues, IL-10$^{-/-}$ mice were found to be even more highly susceptible to *S. mansoni* infection than IL-4$^{-/-}$ mice, despite the presence of Th2 cytokines, suggesting that IL-10 plays an important immunoregulatory role during infection [222]. Hence, the impaired production of IL-10 by MLN cells stimulated with SEA presents another factor that could explain the exacerbated mortality in B cell-specific IL-4Rα deficient mice.

The expansion and differentiation of cytokine producing T cells has been shown to be largely dependent on B cells [90,96,106]. In our study, we found no significant difference in the absolute number of CD3^{+}CD4^{+} T cells between all the mutant mouse strains. However, the expansion of follicular helper T cells (T_{FH}) in the secondary lymphoid tissue was significantly impaired in *mb1*^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/-}$ mice compared to littermate control mice. T_{FH} cells are crucial for lymphoid tissue organisation as they assist in germinal center formation and promote antibody responses, including isotype switching [59,60,62]. In a study by Lin and colleagues, deficiency in B cells was shown not to alter the differentiation of antigen-specific T cells and expression of activation markers CD69 and CD44 [96]. Likewise, the expansion of effector CD4^{+} T cells was not hindered in B cell-specific IL-4Rα deficient mice whereas IL-4Rα$^{-/-}$ mice displayed significantly reduced absolute numbers of effector CD4^{+} T cells compared to littermate control mice. Importantly, the lack of IL-4Rα expression on B cells resulted in abrogated intracellular production of Th2 cytokines IL-4 and IL-13 by CD4^{+} T cells after restimulation with PMA/Ionomycin *ex vivo*, suggesting that IL-4/IL-13 responsive B cells are crucial for promoting the expansion of Th2 cells. Indeed, previous studies have shown that the lack of B cells alters the expansion and differentiation of IL-4 producing effector Th2 cells in response to *Hp* infection *in vivo* [90]. Furthermore, B cells mediate the expansion of primary Th2 cells in response to protein antigens delivered with *Nippostrongylus brasiliensis* (*Nb*) [96] and protein antigens delivered with alum [89]. B cells have also been shown to contribute to susceptibility during *Leishmania major* LV 39 infection in Balb/c mice and promote Th2 immunity [97].

IL-4 is a key cytokine mediating isotype switching towards the production of IgG1 and IgE by B cells [253]. As expected, *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice developed a predominant type 1 antibody response characterised by the production of IgG2a and IgG2b whereas the production of type 2 antibody isotypes was abrogated compared to littermate control mice. Although there was no significant difference in the absolute number of CD19^{+}B220^{+} B cells recruited into the MLN of infected mice, the number of follicular (FO) B cells was significantly reduced in both *mb1*^{cre}IL-

4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$mice compared to littermate control mice. Conversely, the number of marginal zone (MZ) B cells was significantly increased in B cell-specific IL-4Rα deficient mice and remained similar between global IL-4Rα knock-out mice and littermate control mice. FO B cells are found within germinal centres where they form tight physical contact with T cells, thus ensuring optimal T cell proliferation [278]. B cells have been shown to produce Th2 cytokines in response to *Hp* infection *in vivo* [90,106]. The number of IL-4 and IL-13 producing B cells was reduced in both *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice after restimulation of MLN cells with PMA/Ionomycin compared to the littermate control mice. Interestingly though, the absolute number of IFN-γ and TNF-α producing B cells was not concomitantly increased in both B cell-specific IL-4Rα mice and IL-4Rα$^{-/-}$ compared to control mice, suggesting that abrogating IL-4Rα expression on B cells alters both the secretion of Th1 and Th2 cytokines *in vitro*. This contradicts previously published data where B cells generated in the presence of Th2 cells and antigen up-regulated the expression of Th2 cytokines while restricting the production of Th1 cytokines [106]. Expression of activation markers MHC II and CD80 was attenuated in B220^{+} B cells from both B cell-specific IL-4Rα deficient mice and IL-4Rα$^{-/-}$ mice compared to littermate control mice (data not shown). MHC II dependent cognate interactions between B and T cells have been shown to be crucial for the development of optimal Th2 immunity to *Hp* infection [90]. Therefore, IL-4/IL-13 responsive B cells are important for maintaining optimal cellular immunity during infection with *S. mansoni*.

S. mansoni eggs trapped in the host liver and intestine drive CD4^{+} T cell-dependent granulomatous pathology [217,219]. Infected *mb1*^{cre}IL-4Rα$^{-/lox}$ mice developed augmented granuloma formation, increased hepatocellular damage, and similar liver fibrosis to littermate controls. Consistent with previously published data, infected IL-4Rα$^{-/-}$ mice had impaired granuloma formation and enhanced hepatocellular damage compared to littermate control mice [147,148,190]. Exacerbated liver pathology is a key morbidity factor contributing to mortality in IL-4$^{-/-}$ and IL-13$^{-/-}$ mice alongside endotoxemia [219,279]. Restimulation of hepatocytes with either SEA or mitogen resulted in impaired production of both Th1 and Th2 cytokines in B cell-specific IL-4Rα mice. IL-4 is crucial for limiting tissue pathology induced by unregulated nitric oxide production [279]. Alternatively activated macrophages have also been shown to minimise tissue damage after infection with *S. mansoni* in mice [147]. Inflammatory macrophages characterised by the expression of iNOS were detected in close proximity to *S. mansoni* eggs in both *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice while they were not detected at all in littermate control mice. This was further corroborated by flow cytometric analysis of iNOS and Ym-1 expression by liver cells, where CD11b^{+}MHCII^{+} macrophages from *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice displayed a shift towards classical activation compared to littermate control mice.

Maintaining the integrity of the gut is important for surviving *S. mansoni* infection. Mice deficient in IL-4/IL-13 responsive macrophages and IL-4Rα$^{-/-}$ mice fail to regulate intestinal pathology and had massive inflammation in the gut lumen with leaking of LPS into the bloodstream, resulting in endotoxemia and septic shock [147,190,218]. In our study, mice deficient in IL-4Rα expression

specifically on B cells presented with severe gut inflammation indicated by bigger granulomas around the parasite eggs and increased infiltration of inflammatory cells similar to that observed in the highly susceptible IL-4Rα$^{-/-}$ mice. Therefore, these data suggest that severe gut inflammation may have contributed to heightened susceptibility of B cell-specific IL-4Rα deficient mice.

Previous studies have demonstrated that B cell-deficient mice are susceptible to acute schistosomiasis due to accentuated granuloma formation and intensive fibrosis [232,233]. It was further shown that abrogating the expression of FcRγ resulted in exacerbated tissue pathology during both the acute and chronic phases of infection [233]. In this study, we compared mortality between B cell-deficient mice and mice lacking expression of IL-4Rα specifically on B cells to determine the factors contributing to extensive susceptibility to *S. mansoni* infection. We found that *mb1*^{cre}IL-4Rα$^{-/lox}$ mice rapidly succumbed to *S. mansoni* infection compared to µMT mice and displayed 100% mortality by 10 weeks post-infection. In contrast, µMT mice survived the acute phase of infection without mortality and began to dramatically die during the chronic stage of infection, with 50% of mice succumbing to infection in one week. B cell deficient mice had extensive liver damage compared to *mb1*^{cre}IL-4Rα$^{-/lox}$ mice as indicated by augmented granuloma formation and hepatic fibrosis. Consistent with previously published data, MLN cells from µMT mice stimulated with SEA failed to produce IL-4 and IL-10 while the production of IL-5 and IFN-γ was comparable to littermate control mice [233]. Likewise, cells from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice had decreased production of IL-4, IL-5 and IL-10 after antigen specific restimulation, while IFN-γ production was similar to that of littermate control mice. It is interesting to note that B cell deficient mice amplified the production of IL-10 in response to mitogenic stimulation compared to both *mb1*^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice, suggesting that IL-10 may be playing an immunomodulatory role that results in the delayed susceptibility in these mice. Thus, these data suggest that the ability of B cells to respond to IL-4/IL-13 stimuli and not the absence of B cells is crucial for conferring protection against acute schistosomiasis.

Studies have demonstrated that the host immune response is gradually attenuated as the infection progresses towards chronicity and this is indicated by the decreased size of newly formed granulomas and T cell hypo-responsiveness to egg antigen. Initially, CD8$^+$ suppressor cells [229,271] and cross-regulatory cytokines produced by CD4$^+$ T cells [230,231,270] were thought to be involved in the mechanism down-regulating the host immune response during the chronic stages. However, a study by Yap and colleagues demonstrated that mice deficient in functional CD8$^+$ T cells or IFN-γ are still capable of down-modulating egg-induced pathology during chronic stages of infection [280]. However, mice deficient in B cells failed to down-regulate granuloma formation although T cell responsiveness was unaltered during the chronic stages of infection [232,233]. A recent study by Fairfax and colleagues showed that interference with IL-10R responsiveness results in severe chronic disease characterised by portosystemic shunting of the eggs to the heart and lungs due to increased hepatic-portal blood pressure [221]. In our study we found that B cell-specific IL-4Rα deficient mice failed to down-regulate liver and intestinal

granuloma size during the chronic stages of infection compared to littermate control mice. At 16 weeks post-infection, production of the immunoregulatory cytokine IL-10 was significantly reduced in both *mb1*^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice compared to littermate control mice, while at 24 weeks post infection B cell-specific IL-4Rα deficient mice displayed dramatic up-regulation of IL-4, IL-6, IL-17 and IFN-γ. Importantly, *S. mansoni* eggs were found in the lungs and spleen of both *mb1*^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/lox}$ mice, indicating the development of severe disease. In humans, hepatosplenic disease reflects a failure to modulate the immune response and causes mortality if left untreated [220].

B cells make large quantities of Th2 cytokines after being cultured in the presence of Th2 cells and antigen *in vitro* [105,106]. The lack of IL-4 producing B cells during *Hp* infection did not hamper parasite clearance, indicating that development of protective immunity occurs independently of B cell-derived IL-4 [90]. Consistent with this data, we found in our study that mice lacking IL-4 production specifically by B cells (B IL-4$^{-/-}$) developed slightly reduced granuloma formation, normal fibrosis and hepatocellular damage, unaltered antigen-specific cytokine production and a CD4^{+} T cell activation profile similar to wild-type (WT) mice. Production of Th2 cytokines by CD4^{+} T cells stimulated with PMA/Ionomycin *ex vivo* was not affected in mice lacking IL-4 expression by B cells, although IFN-γ production was significantly reduced compared to WT mice. Liu and colleagues showed that B cell-derived IL-4 does not play an essential role in Th2 cell development [96]. B cell responses were sufficient in B IL-4$^{-/-}$ mice as indicated by isotype switching towards the production of IgG1 and total IgE, recruitment of B220^{+} B cells into the lymphoid tissue, expansion of FO B cells and similar levels of cytokine production compared to WT mice. However, the expression of the activation markers MHC II and CD80 was abrogated in both B-IL-4$^{-/-}$ and IL-4$^{-/-}$ mice compared to WT mice. In a study by Wojciechowski and colleagues, IL-2 and TNF-α derived from B cells were found to mediate protection to *Hp* infection and contribute to the development of Th2 cells. Thus, B cell-derived IL-2 and TNF-α may be involved in the mechanism conferring protection during *S. mansoni* infection in mice.

In conclusion, we have demonstrated that IL-4/IL-13 responsive B cells are required for surviving acute schistosomiasis and contribute towards limiting organ injury. Moreover, expression of IL-4Rα on B cells is crucial for the development of optimal cellular and humoral immunity during infection. Mice lacking IL-4Rα expression specifically on B cells failed to down-modulate granuloma formation during the chronic stages of infection, exhibited T cell hyper-responsiveness at the later stages of infection and developed severe disease characterised by shunting of the eggs to the lungs and spleen. Lastly, B cell-derived IL-4 was shown not to be contributing towards the generation of host protective immune responses to *S. mansoni* infection.

CHAPTER 4

CHAPTER 4

IL-4/IL-13 responsive CD11c^{+} dendritic cells are required for protection against acute schistosomiasis and modulate T helper cell immunity.

Hlumani Ndlovu[1#], Natalie Niuweinhuizen[1], William Horsnell[1], and Frank Brombacher[1*]

[1] International Center for Genetic Engineering and Biotechnology (ICGEB), Cape Town Component and Institute of Infectious Diseases and Molecular Medicine (IIDMM), Division of Immunology, University of Cape Town, Cape Town, South Africa ,

Abstract

Development of IL-4Rα-dependent Th2 immunity and granuloma formation is crucial for host survival during acute Schistosomiasis. Mice deficient in IL-4Rα expression on macrophages quickly succumb to *S. mansoni* infection due to increased liver and gut pathology. Furthermore, pan-T cell-specific IL-4Rα deficient mice (iLck^{cre}IL-4Rα$^{-/lox}$) were highly susceptible to acute schistosomiasis despite controlling gut inflammation, indicating a requirement for IL-4/IL-13 responsive non-CD4^{+} T cells during protective responses. Other possible IL-4/IL-13 responsive cellular populations involved in conferring protection against acute schistosomiasis are yet to be identified. Hence, in this study, we investigated the role of IL-4Rα signalling on CD11c^{+} dendritic cells (DCs) during *S. mansoni* infection using a novel mouse (CD11c^{cre}IL-4Rα$^{-/lox}$) which lacks IL-4Rα expression on CD11c^{+} dendritic cells and alveolar macrophages. CD11c^{cre}IL-4Rα$^{-/lox}$ Balb/c mice developed a severe wasting disease that resulted in 100% mortality by 13 weeks post-infection. Mortality occurred despite controlled liver and intestinal pathology and was associated with increased serum TNF-α levels. Abrogation of IL-4Rα expression on DCs impacted on the activation of CD4^{+} T cells, resulting in reduced numbers of effector CD4^{+} T cells and impaired production of IL-5, IL-13 and IFN-γ by CD4^{+} T cells *ex vivo*. In addition, DCs and macrophages had significantly increased iNOS expression, a marker for classical activation. Therefore, IL-4/IL-13 responsive CD11c^{+} DCs are required for surviving acute schistosomiasis and play an important role in orchestrating cellular immunity.

Keywords: Dendritic cells, acute, schistosomiasis, T helper; mice.

4.1 Introduction

Schistosomiasis is an important parasitic disease infecting more than 200 million people, mostly in the developing countries, and causing an estimated 280 000 deaths per annum in sub-Saharan Africa alone [265,266]. During infection in both humans and mice, *S. mansoni* eggs trapped in the host tissue (particularly in the liver) induce a strong granulomatous inflammatory response that is accompanied by augmented production of Th2 cytokines, eosinophilia, goblet cell hyperplasia and increased IgG1 and IgE production [131,217,281]. Infection of IL-$4^{-/-}$ and IL-4/IL-$13^{-/-}$ mice with *S. mansoni* revealed a crucial protective role for Th2 cytokine mediated responses and granuloma formation [148,219,222]. IL-4 and IL-13 are key Th2 cytokines that signal though the common receptor chain, the IL-4 receptor alpha (IL-4Rα) [167,168]. IL-4 exclusively signals through the type 1 receptor composed of IL-4Rα and the common gamma chain (γc) while both IL-4 and IL-13 signal through the type II receptor comprising of IL-4Rα and IL-13Rα1 chains [167,168]. Mice deficient in IL-4Rα signalling quickly succumb to *S. mansoni* infection due to impaired granuloma formation, impaired Th2 polarization, increased liver inflammation and destruction of the gut integrity that results in endotoxemia and septic shock [147,148,190]. Therefore, IL-4 and IL-13 responsiveness plays a key role in down-regulating organ injury induced by *S. mansoni* eggs.

Development of cell-specific IL-4Rα mutant mouse strains has proven to be an invaluable tool for dissecting the mechanisms conferring protection or susceptibility to the host during *S. mansoni* infection. Previous studies from our laboratory have demonstrated a pivotal role for IL-4/IL-13 responsive macrophage in host protective responses during acute Schistosomiasis [147]. Mice deficient in IL-4Rα expression on macrophages ($LysM^{cre}$IL-$4R\alpha^{-/lox}$) were extremely susceptible to *S. mansoni* infection due to increased hepatocellular damage and intestinal inflammation that led to endotoxemia and septic shock [147]. Furthermore, using mice that lacked IL-4Rα expression on pan-T cells ($iLck^{cre}$IL-$4R\alpha^{-/lox}$), it was shown that IL-4Rα responsive non –$CD4^+$ T cells contribute to resistance against acute schistosomiasis by controlling excessive liver inflammation [190]. Interestingly, the candidate IL-4Rα responsive cellular populations responsible for conferring protection against *S. mansoni* infection are yet to be identified. Here, we postulate that IL-4Rα responsive dendritic cells contribute to host protection to *S. mansoni* infection via amplification of the Th2 immune response.

Dendritic cells (DCs) are the most specialised antigen presenting cells (APCs), capable of recognising and processing foreign antigen, migrating to the lymph nodes and efficiently initiating T cell activation [282,283,284]. The ability of DCs to orchestrate Th1/Th17 immunity in response to bacterial and viral pathogens is well established, whereas there is a paucity of understanding about their activation and functional capability in a Th2 setting induced by helminth infection [217,285]. However, recent studies conducted using CD11c-diphtheria toxin receptor mice to deplete $CD11c^+$ DCs, have demonstrated a critical role played by $CD11c^+$ DCs in inducing Th2 immunity during *S. mansoni* infection [286]. Furthermore, bone marrow derived dendritic cells

(BMDCs) have been shown to respond to IL-4 by up-regulating alternative activation markers such as Ym1/2 and RELMα, and abrogation of IL-4Rα expression on DCs resulted in impaired IFN-γ production in both Th1 and Th2 settings [287]. Interestingly, IL-4 has been shown to induce the production of IL-12p70 by bone marrow derived dendritic cells (BMDCs) stimulated with bacterial LPS or CpG *in vitro* [288,289,290,291].

Here, we investigated whether IL-4Rα signalling on DCs plays a role in rendering the host protected or susceptible during *S. mansoni* infection, utilising a transgenic mouse strain ($CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$) where IL-4Rα expression was interrupted specifically on $CD11c^{+}$ DCs. $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were highly susceptible to *S. mansoni* infection regardless of their ability to down-regulate liver and gut inflammation. Furthermore, the lack of IL-4Rα expression on DCs did not inhibit production of key Th2 cytokines IL-4 and IL-5 after restimulation of total lymph nodes cells with either SEA or α-CD3 *ex vivo*. Interestingly though, production of both Th1 and Th2 cytokines by $CD4^{+}$ T cells was impaired in $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice. Finally, macrophage and DCs activation appeared to be skewed towards classical activation as indicated by elevated levels of iNOS. Together, these data reveal that IL-4Rα signalling on DCs is important for resistance to acute schistosomiasis and plays a major role in driving T cell responses.

4.2 Materials and Methods

Generation and genotyping of $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ Balb/c mice. $CD11c^{cre}$ mice were intercrossed with $IL\text{-}4R\alpha^{lox/lox}$ Balb/c mice [147]. These mice were further mated with homozygous $IL\text{-}4R\alpha^{-/-}$ Balb/c mice [248] to generate hemizygous $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice. Hemizygous littermates ($IL\text{-}4R\alpha^{-/lox}$) were used as wild-type controls in all experiments. Mice were genotyped as described previously [147,248]. All mice were housed in specific pathogen-free barrier conditions in individually ventilated cages at the University of Cape Town biosafety level 2 animal facility. Experimental mice were age and sex matched and used between 8-12 weeks of age.

Ethics statement. This study was performed in strict accordance with the recommendations of the South African national guidelines and University of Cape Town practice of laboratory animal procedure. All mouse experiments were performed according to the protocols approved by the Animal Research Ethics Committee of the Faculty of Health Sciences, University of Cape Town (protocol number: 010/041). Efforts were made to minimise and reduce suffering of animals.

Live *S. mansoni* infection of mice. Mice were percutaneously infected with 100 live cercariae (acute infection) of a Puerto Rican strain of *S. mansoni* obtained from infected *Biomphalaria glabrata* (a generous gift from Adrian Mountford, York, UK). The mice will be monitored weekly until 7 weeks post-infection.

Footpad model. Mice were challenged by injection of 2 500 *S. mansoni* eggs into the right hind footpad and killed 7 days post-challenge.

Antibodies and flow cytometry. The following antibodies comprising the B cell antibody panel were used: B220-V500, CD19-PerCP Cy5.5, CD23-PE, CD21-APC, CD24-PE Cy7, CD80-V450, MHCII-FITC and IgM-Biotin (BD Bioscience, Erembodegem, Belgium). T cells panel consisted of the following antibodies: CD4-PerCP, CD3-AlexaFluor 700, CD62L-V500, CD44-FITC, CD28-PE, CXCR5-V450 and CD278-Biotin (BD Bioscience, Erembodegem, Belgium). Cells were acquired on a FACS Fortessa machine (BD Immunocytometry system, San Jose, CA, USA) and data was analyzed using Flowjo software (Treestar, Ashland, OR, USA).

Macrophage and dendritic cell staining. Activation profile of macrophage ($CD11b^{+}MHCII^{+}$) and DCs ($CD11c^{+}MHCII^{+}$) was analysed by flow cytometry. Classically activated macrophages and DCs were detected by staining for intracellular expression of iNOS using rabbit anti-mouse iNOS antibody (Abcam) with goat anti-rabbit PE (Abcam). Alternatively activated macrophages and DCs were detected by detecting Arginase 1 and Ym-1-Biotin using goat anti-mouse arginase (Santa Cruz Biotechnology) with donkey anti-goat PE (Abcam) and Strep-APC respectively. Staining specificity was verified by appropriate isotype-matched antibody controls and compensation performed with single-stain samples before acquiring the multi-coloured samples. Acquisition was performed using a FACSCalibur (BD Immunocytometry Systems).

Intracellular cytokine staining. For detection of intracellular cytokines, MLN cells from *S. mansoni* infected mice or MST from *S. mansoni* eggs injected mice were plated at 1×10^6 cells/well and stimulated at 37ºC for 4 hours with 50 ng/ml phorbal (myristate acetate), 250 ng/ml ionomycin and 200 µM monensin in IMDM/10% FCS (all purchased from Sigma-Aldrich). Cells were stained with extracellular markers (CD4 Biotin-APC, B220 FITC or CD19 PercP), fixed for 30 min on ice in 2% (w/v) paraformaldehyde and permeabilised with 0.5% saponin buffer and stained with PE-labelled anti-mouse IL-4, IL-5, IL-10 and IFN-γ for 15 min. Acquisition was performed using a FACSCalibur (BD Immunocytometry Systems, San Jose, CA, USA) and data were analysed using FlowJo software (Treestar, Ashland, OR, USA).

Cell preparation and *ex vivo* restimulation. Single cell suspensions were prepared by pressing the draining lymph nodes through 70 µM cell-strainers. Cells were resuspended in complete DMEM (Gibco) supplemented with 10% FCS (Gibco) and penicillin and streptomycin (100 U/ml and 100 µg/ml, Gibco). The cells were cultured at 2×10^6 cells/ml in 48-well plates coated with α-CD3 (20 µg/ml) or soluble egg antigen (SEA, 20 µg/ml) and incubated at 37ºC in a humidified atmosphere containing 5% CO_2. Supernatants were collected after 72 h and cytokines were

measured by ELISA. Quantities of IL-4, IL-10 and IL-13 were measured by sandwich ELISA as previously described [248].

Enzyme Linked Immunosorbent Assays (ELISAs). Cytokines in supernatant were measured by sandwich ELISA as previously described [248]. For antibody ELISAs, blood was collected in serum separator tubes (BD Bioscience, San Diego, CA) and serum was separated by centrifugation at 8 000 ×g for 10 min at 4ºC. Titres of SEA-specific IgG1, IgG2a, IgG2b and total IgE were determined as previously described [248]

Hydroxyproline assay. Hydroxyproline content as a measure of collagen production was determined using a modified protocol [276]. Briefly, weighed liver samples were hydrolysed and added to a 40 mg Dowex/Norit mixture. The supernatants was neutralised with 1% phenolphthalein and titrated against 10 M NaOH. An aliquot was mixed with isopropanol and added to chloramine-T/citrate buffer solution (pH 6.5). Erlich's reagent was added and absorbance was read at 570 nm. Hydroxyproline levels were calculated using 4-hydroxy-L-proline (Calbiochem) as a standard, and results were expressed as µmoles hydroxyproline per weight of tissue that contained 10^4 eggs.

Histology. Liver and gut samples were fixed in 4% (v/v) formaldehyde in phosphate buffered saline, embedded in wax and processed. Sections (5-7 µm) were stained with hematoxylin and eosin (H&E) and analine blue solution (CAB) and counterstained with Wegert's hematoxylin for collagen staining. Micrographs of liver granuloma were captured using a Nikon 5.0 mega pixel colour digital camera (DCT DS-SMc).The diameter of each granuloma containing a single egg was measured with the ImageJ 1.34 software. An average of 25 granulomas per mouse was included in the analyses.

Statistics. Statistical analysis was conducted using GraphPad Prism 4 software. Data was calculated as mean ± SD. Statistical significant was determined using the unpaired Student's *t* test or 2-way ANOVA with Bonferroni's post test, defining differences to IL-4Rα$^{-/lox}$ mice as significant (*,$p \leq 0.05$; **,$p \leq 0.01$; ***,$p \leq 0.001$). (Prism software; http://www.prism-software.com).

4.3 Results

IL-4/IL-13 responsive CD11c^{+} dendritic cells are essential for survival in acute schistosomiasis

In order to investigate the role of IL-4Rα responsive DCs during *S. mansoni* infection, CD11c^{cre}IL-4Rα$^{-/lox}$ Balb/c, IL-4Rα$^{-/-}$ and IL-4Rα$^{-/lox}$ mice were infected with 80 live *S. mansoni* cercariae and mortality was monitored over a 16 weeks period (Fig 4.1A). Although IL-4Rα$^{-/lox}$ littermate control mice survived infection with little mortality and morbidity, all IL-4Rα$^{-/-}$ mice rapidly succumbed to infection by 10 weeks post-infection, and CD11c^{cre}IL-4Rα$^{-/lox}$ mice succumbed to infection by 13 weeks post-infection ((Fig 4.1A). The high mortality rate observed in IL-4Rα$^{-/-}$ mice correlated with increased morbidity, indicated by severe body weight loss that began shortly after 6 weeks post-infection, coinciding with egg laying by the parasites (Fig 4.1B). Initially, infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice began to lose weight at 6 weeks post-infection, but appeared to recover around 9 weeks post-infection (Fig 4.1B). Nevertheless, they began to drastically lose weight again as they entered the more chronic stages of infection compared to IL-4Rα$^{-/lox}$ littermate control mice (Fig 4.1B). These data suggests that IL-4/IL-13 responsive DCs are required for protection against acute schistosomiasis in mice.

To investigate the mechanism responsible for increased susceptibility in CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice, sera from infected mice were analysed for levels of aspartate transaminase (AST) and key cytokines IL-4 and TNF-α. Surprisingly, the levels of AST were similar between DC-specific IL-4Rα-deficient mice and IL-4Rα$^{-/lox}$ littermate control mice (Fig 4.1C), indicating normal liver function. In contrast, levels of AST were significantly increased in sera from IL-4Rα$^{-/-}$ mice compared with IL-4Rα$^{-/lox}$ littermate control mice, indicating increased hepatocellular damage (Fig 4.1C). The levels of serum TNF-α were significantly elevated in both CD11c^{cre}IL-4Rα$^{-/lox}$ mice and IL-4Rα$^{-/-}$ mice, while the levels of key protective cytokine IL-4 were reduced in both mutant strains compared with IL-4Rα$^{-/lox}$ littermate control mice (Fig 4.1D and E). These data show that elevated serum TNF-α level was associated with high mortality rates in mutant mouse strains.

Another cause of death during *S. mansoni* infection in mice is septic shock caused by leaking of LPS into the bloodstream due to destruction of gut integrity [147,219]. Although both IL-4Rα mutant mice succumbed to *S. mansoni* infection, examination of organ pathology clearly showed that distinct mechanisms were responsible for mortality. Small intestines from IL-4Rα$^{-/-}$ mice were highly inflamed (Figure 4.1F). Furthermore, mice suffered from multiple organ defects, indicated by severe atrophy of the spleen and liver (Figure 4.1F). In contrast, small intestines from CD11c^{cre}IL-4Rα$^{-/lox}$ mice showed little or no inflammation. However, mice presented with severe colon problem resulting in failure to excrete the faeces (Figure 4.1F).

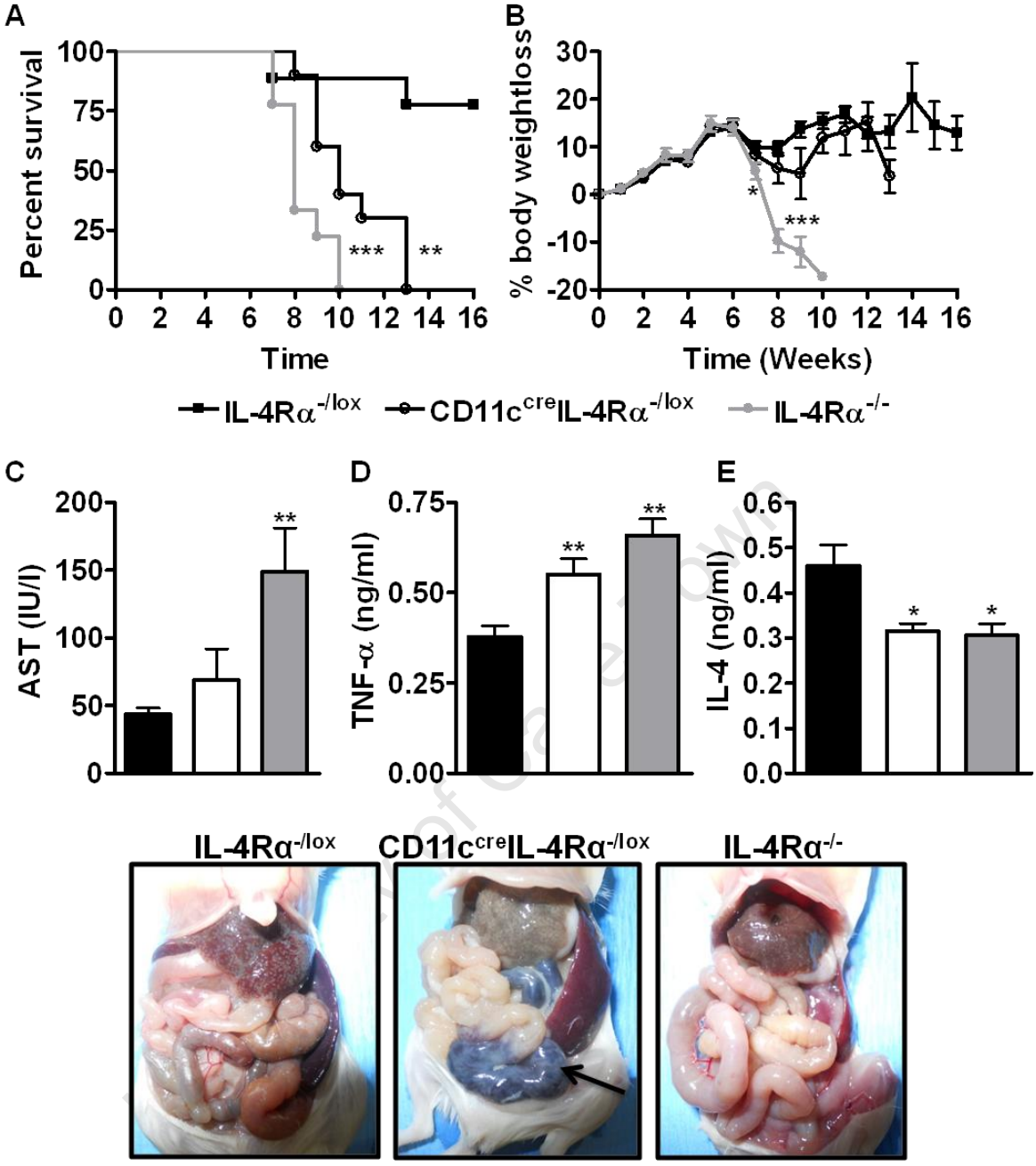

Figure 4.1: IL-4Rα responsive CD11c^{+} dendritic cells are required for protection against acute schistosomiasis in mice.
IL-4Rα$^{-/lox}$, CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 80 live *S. mansoni* cercariae and survival monitored over a 16 week period. (**A**) Survival kinetics of mice infected percutaneously with 100 cercariae. (**B**) Percent body weight loss monitored on a weekly basis. (**C**) Serum levels of aspartate transaminase. (**D-E**), Serum levels of TNF-α and IL-4 was determined by ELISA. Data represents two independent experiments (n = 8-10). Survival curves were compared using Logrank test. *p< 0.05 and **p< 0.01 vs IL-4Rα$^{-/lox}$ mice.

Unaltered cytokine production by total LN cells from CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

Cellular responses were examined to determine whether abrogation of IL-4Rα responsiveness on CD11c^{+} DCs affects the development of Th2 immune responses during *S. mansoni* infection. Total draining lymph node cells were restimulated with SEA and α-CD3 and cytokines were

measured in the supernatants. The production of antigen specific Th2 cytokines (IL-4, IL-5 and IL-10) by total MLN cells was similar in CD11^{cre}IL-4Rα$^{-/lox}$ mice and littermate controls, although IL-13 production was reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Fig 4.2A). Restimulation of total MLN cells with α-CD3 resulted in similar levels of IL-4, IL-5, IL-13 and IL-10 in CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice, but increased level of Th1 cytokine IFN-γ in CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Fig 4.2B). These data suggests that Th1 and Th2 cytokines detected during antigen-specific and mitogen restimulation of total MLN cells may have come from non-CD4^{+} T cell source.

Single cell suspensions were prepared from mesenteric lymph node and cells were stained for flow cytometry. The total number of MLN cells was significantly reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice (Fig 4.2C). This corresponded with a dramatic decrease in the total number of CD4^{+} T cells in CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Fig 4.2D), although the percentages of CD4^{+} T cells were similar between the CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice (data not shown). Likewise, the total number of effector CD4^{+} T cells was significantly reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice (Fig 4.2E). This suggests that IL-4/IL-13 responsive DCs are required for promoting CD4^{+} T cell responses during *S. mansoni* infection. Cytokine secretion by LN CD4^{+} T cells was also determined by intracellular flow cytometry. Although the percentage of CD4^{+} T cells secreting IL-4 and IL-10 was similar between CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate controls, the percentage of CD4^{+} T cells producing IL-5 and IL-13 was significantly reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Fig 4.2F). In addition, production of Th1 cytokine IFN-γ by CD4^{+} T cells was also reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice, suggesting that IL-4/IL-13 responsive DCs are required to initiate and maintain cytokine production by CD4^{+} T cells.

Reduced recruitment and activation of B cells in CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

Depletion of CD11c^{+} DCs has been shown to affect B cells development during *S. mansoni* infection [286]. The levels of type 2 antibodies (SEA-specific IgG1 and total IgE) and type 1 antibodies (SEA-specific IgG2a and IgG2b) were similar between infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice (Fig 4.3A). In contrast, IL-4Rα$^{-/-}$ mice displayed elevated serum titres of SEA-specific type 1 antibodies and reduced type 2 antibodies (Fig 4.3A). Therefore, these data suggest that IL-4/IL-13 responsive DCs are not important for the development of humoral immune responses. Flow cytometry was performed to explore the role of IL-4Rα deficient DCs in B cell activation. The total number of CD19^{+} B cells was reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice (Fig 4.3B). Expression of activation markers MHCII and CD86 by CD19^{+} B cells was also reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared with littermate control mice (Fig 4.3C). Furthermore, there was no difference in the secretion of IL-4 and IL-5 by CD19^{+} B cells from CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice, except for the reduced secretion of IL-13 and IFN-γ (Fig 4.3D). Together, these data suggest that IL-4/IL-13

responsive DCs may affect activation of B cells, possibly via activation of $CD4^+$ T cells and are crucial for the production of IL-13 and IFN-γ by $CD4^+$ T cells.

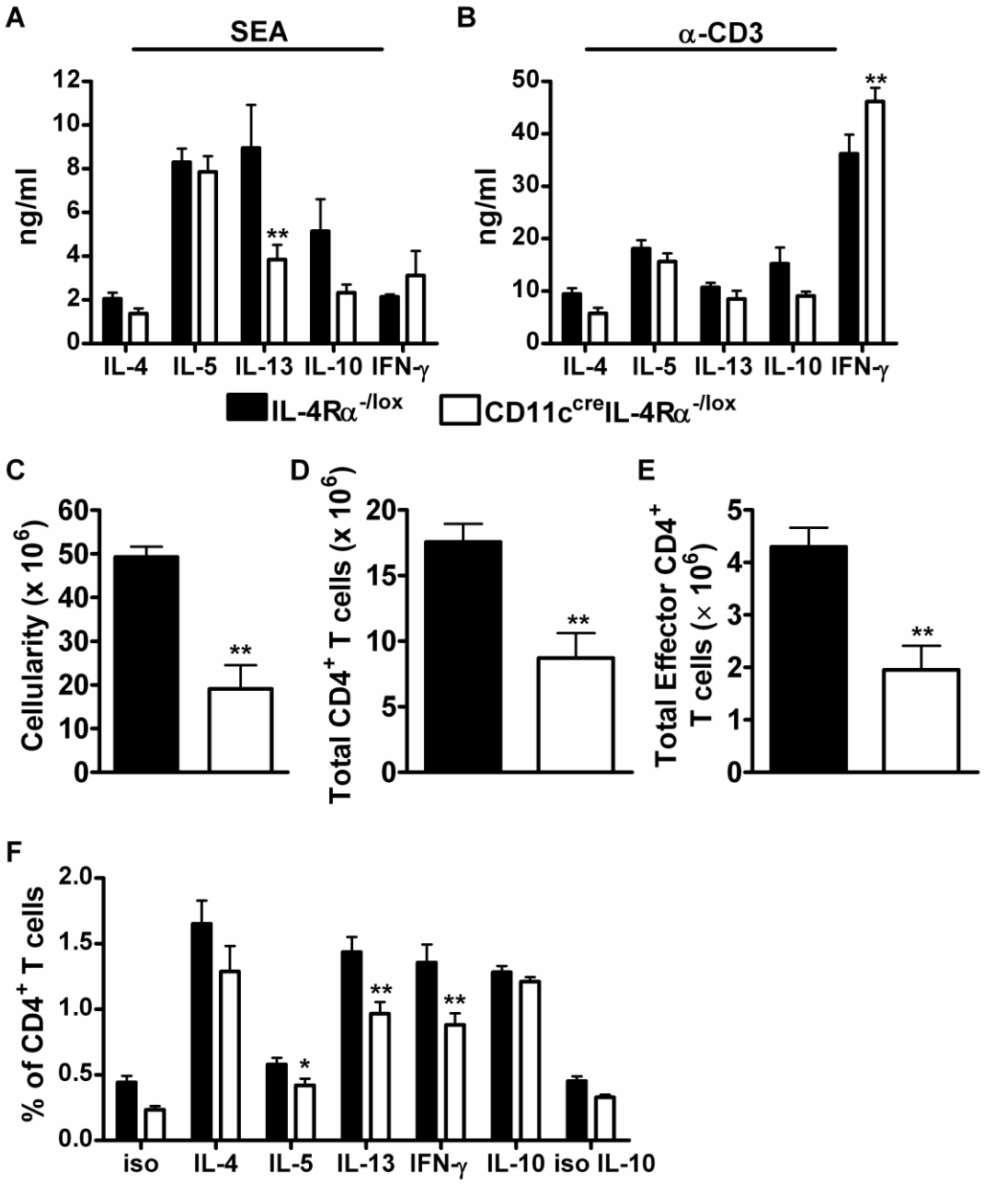

Figure 4.2: Decreased numbers of $CD4^+$ T cells recruited to the draining lymph node of $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice.

Single cell suspension was prepared from mesenteric lymph nodes and cells were stained for flow cytometry. (**A**-**B**) Cytokine production by mesenteric lymph node cells re-stimulated with either SEA or α-CD3 (mean ± SEM). (**C**) Total cell numbers in the draining lymph node. Total numbers of $CD4^+$ T cells (**D**) and effector $CD4^+$ T cells ($CD4^+CD44^{hi}CD62L^{lo}$, **E**) in the lymph node. (**F**) Intracellular cytokine production by MLN $CD4^+$ T cells re-stimulated with 25 ng/ml PMA and 1 µg/ml ionomycin *ex vivo* in the presence of monensin. Data are representative of two independent experiments. **$p< 0.01$ vs $IL\text{-}4R\alpha^{-/lox}$ mice.

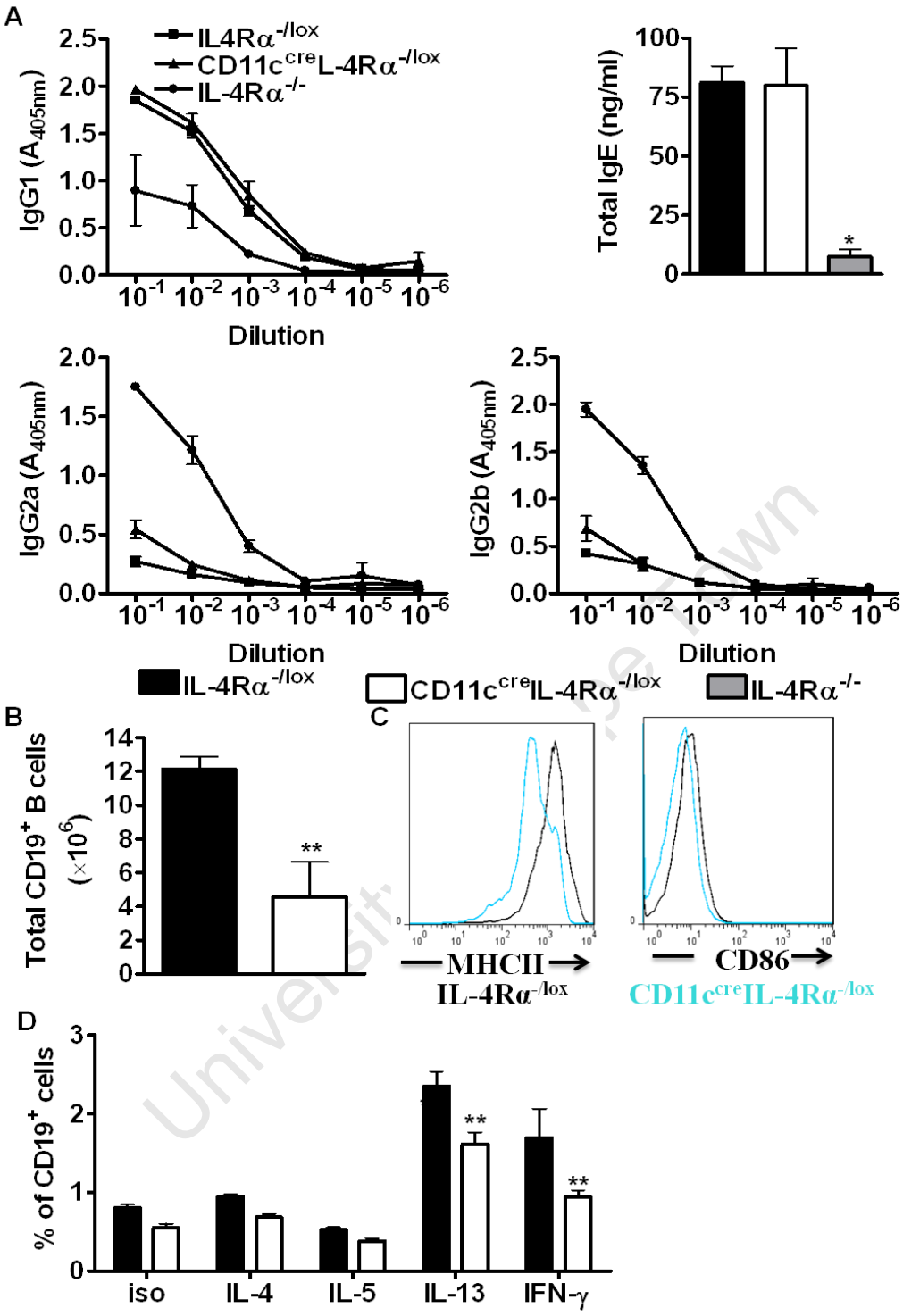

Figure 4.3: Antibody production and lymph node cell cytokine production in CD11c^{+} dendritic cell-specific IL-4Rα deficient mice.

IL-4Rα$^{-/lox}$, CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 100 live *S. mansoni* cercariae and analysed 7 weeks post-infection. (**A**) Serum antibody titres of SEA specific immunoglobulin (IgG) and total IgE were quantified by ELISA. (**B**) Total number of CD19^{+} B cells in the MLN. (**C**), Histograms showing the expression of MHC II and CD86 by CD19^{+} B cells. (**D**) Intracellular cytokine production by MLN CD19^{+} B cells re-stimulated with 25 ng/ml PMA and 1 µg/ml ionomycin *ex vivo* in the presence of monensin. Data represents 2 independent experiments. n = 4-6 mice. *p<0.05, **p<, 0.01 and ***p< 0.001 vs IL-4Rα$^{-/lox}$ mice.

IL-4/IL-13 responsive CD11c^{+} DCs are required for optimal Th2 responses to *S. mansoni* eggs injected into the footpad

To confirm the importance of IL-4/IL-13 responsive DCs in Th2 priming, we utilised a controlled system where *S. mansoni* eggs were injected in the right hind footpad of CD11c^{cre}IL-4Rα$^{-/lox}$ mice, IL-4Rα$^{-/-}$ mice and IL-4Rα$^{-/lox}$ littermate control mice. Antigen-specific (SEA) restimulation of total popliteal lymph node cells (pLN) revealed significantly reduced production of IL-13 in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, but no difference in the production of IL-4 and IL-10, compared to littermate control mice (Fig 4.4A). In global IL-4Rα knock-out mice the levels of Th2 cytokines (IL-4, IL-13 and IL-10) were significantly reduced compared to littermate control mice (Fig 4.4A), while IFN-γ production was significantly increased indicating a shift towards a Th1 immune response (Fig 4.4A). Restimulation of total pLN cells with α-CD3 showed significantly reduced production of IL-4 in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, but production of IL-13 and IL-10 was similar to the littermate control mice (Fig 4.4B). Surprisingly, production of the Th1 cytokine IFN-γ was significantly reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared with littermate control mice (Fig 4.4B). Likewise, IFN-γ production was down-regulated in IL-4Rα$^{-/-}$ mice, concomitantly with the reduced levels of Th2 cytokines IL-4, IL-13 and IL-10. Interestingly, production of IL-4 and IFN-γ by CD19^{+} B cells was abrogated in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice while CD19^{+} B cells from IL-4Rα$^{-/-}$ mice failed to secrete both Th1 and Th2 cytokines following restimulation with PMA/Ionomycin *ex vivo* (Fig 4.4C). Therefore, these data suggests that antigen-specific IL-13 production is dependent on IL-4/IL-13 responsive DCs.

Increased production of INOS by IL-4Rα deficient DCs.

Previously it was shown that bone marrow derived DCs (BMDCs) restimulated with recombinant IL-4 *in vitro* up-regulated the production of Relm-α and Ym-1/2 and had increased mRNA transcripts for *Retnla*, *Clec7a*, *Mrc*1 and *Ccl24* [287], markers previously associated with alternatively activated macrophages [292,293]. We assessed the impact of IL-4Rα deficiency on DCs during live *S. mansoni* infection. The proportion and median of Ym-1 expression by CD11c^{+}MHCII^{+} DCs was similar between CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice (Fig 4.5A). In contrast, expression of iNOS by DCs was significantly up-regulated in IL-4Rα deficient DCs, indicating a shift towards classical activation of DCs (Fig 4.5B). Macrophages from CD11c^{cre}IL-4Rα$^{-/lox}$ mice also displayed a classically activation profile indicated by increased expression of iNOS by CD11b^{+}MHCII^{+} macrophages and decreased Ym-1 expression (Fig 4.5C and D). Finally, the proportion of CD11c^{+}MHCII^{+} DCs producing IL-12 and IL-10 was significantly reduced in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice (Fig 4.5E)..

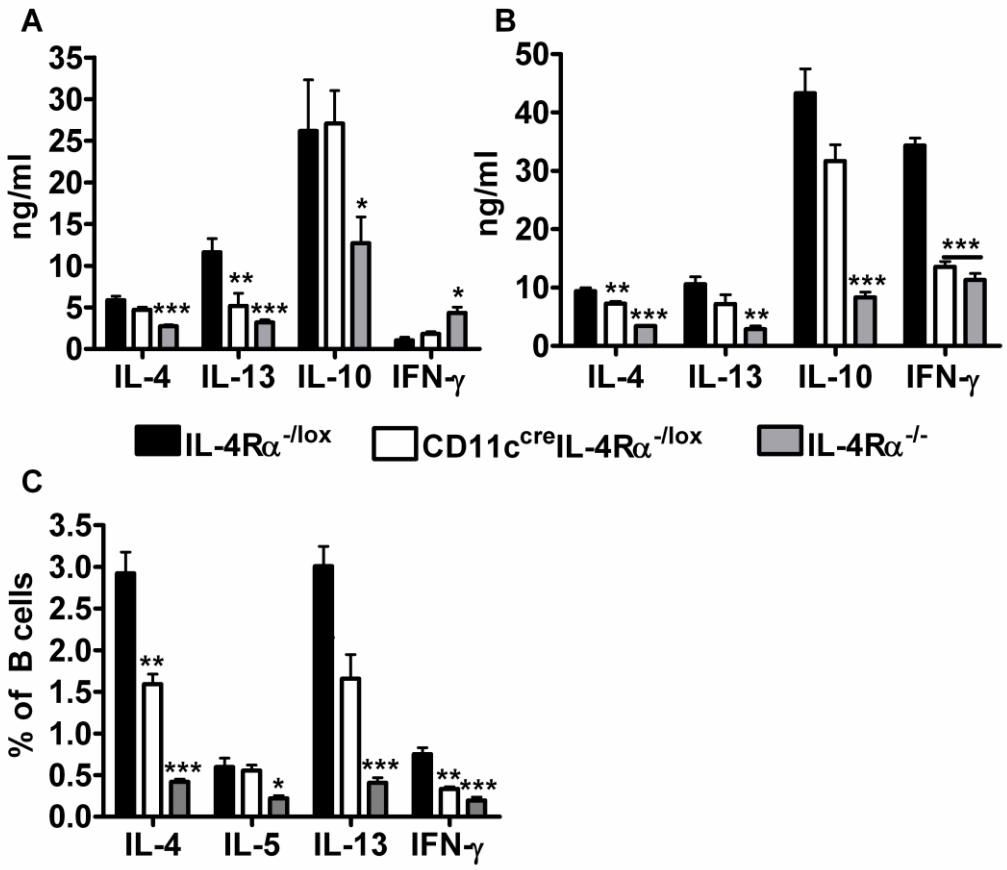

Figure 4.4: Reduced production of Th2 cytokines by total pLN cells.
IL-4Rα$^{-/lox}$, CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were injected with 2 500 *S. mansoni* egg in the right hind footpad and mice were killed 7 post-infection. Single cells suspension of popliteal lymph node (pLN) cells was prepared and cells were *in vitro* restimulated with 20 µg/ml SEA or 20 µg/ml α-CD3 for 72 hrs. Data shown are gated on CD19^{+} B cells. (**A**) Cytokine production by total pLN restimulated with 20 µg/ml SEA. (**B**) Cytokine production by total pLN cells restimulated with 20 µg/ml α-CD3. (**C**) Intracellular cytokine production by CD19^{+} B cells after restimulation with 25 ng/ml PMA and 1 µg/ml ionomycin *in vitro* Data are representative of two independent experiments. n= 6 mice. *p< 0.05, **p< 0.01 and ***< 0.001 vs IL-4Rα$^{-/lox}$ mice.

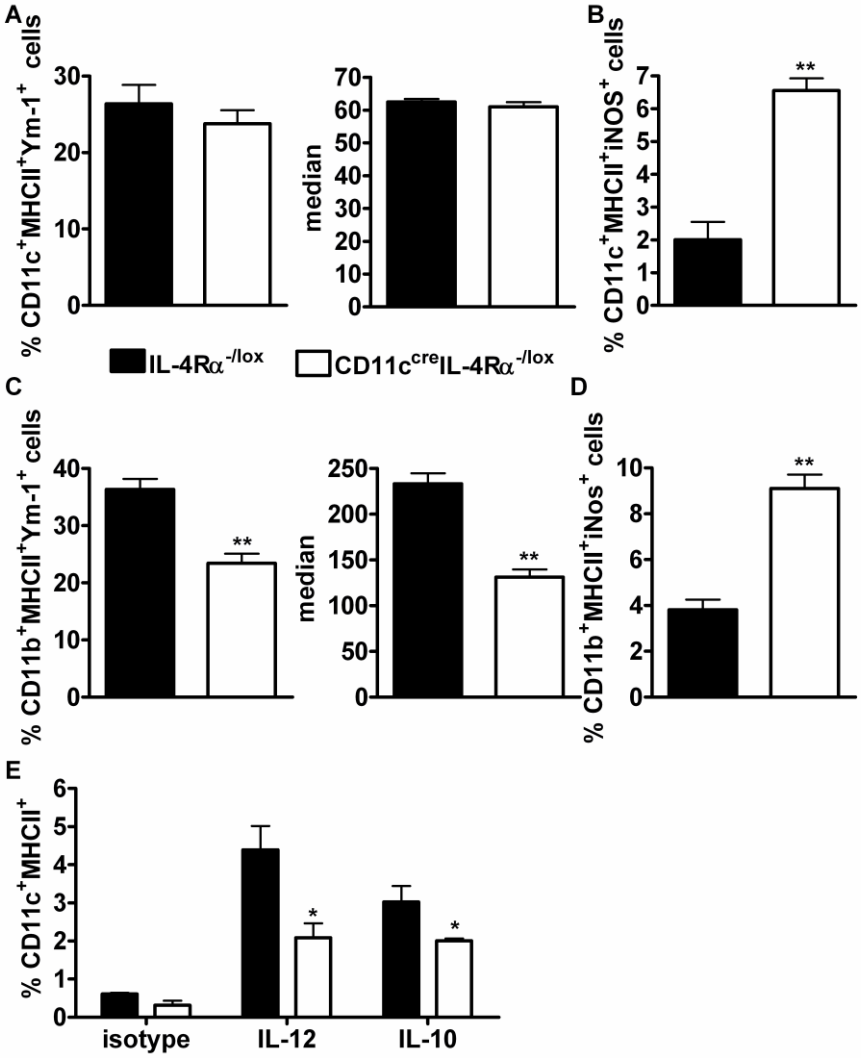

Figure 4.5: Elevated expression of iNOS in CD11c$^+$ dendritic cells deficient in IL-4Rα expression.
Single cell suspensions were prepared from liver tissue and cells were stained for FACS. (**A-B**) Analysis of Ym-1 and iNOS intracellular expression by CD11c$^+$MHCII$^+$ dendritic cells in the liver. (**C-D**) Intracellular expression of Ym-1 and iNos by CD11b$^+$MHCII$^+$ liver macrophages. (**E**) Intracellular cytokine production by CD11c$^+$MHCII$^+$ dendritic cells in the draining mesenteric lymph node after re-stimulation with 25 ng/ml PMA and 1 µg/ml Ionomycin. . Data represents two independent experiments. *p< 0.05 and **p< 0.01 vs IL-4Rα$^{-/lox}$ mice.

CD11c^{cre}IL-4Rα$^{-/lox}$ mice have reduced liver and gut pathology after *S. mansoni* infection

IL-4Rα signalling is critical for controlling granulomatous pathology during *S. mansoni* infection [147,186,190]. Previously, abrogation of IL-4Rα signalling on macrophages resulted in augmented granuloma formation, increased hepatocellular damage indicated by elevated serum AST, and unaltered hydroxyproline production, thus suggesting that IL-4/IL-13 responsive macrophages are essential for down-modulating liver pathology [147]. Therefore, we investigated the contribution of IL-4/IL-13 responsive dendritic cells in granulomatous liver pathology induced by *S. mansoni* eggs. Histological examination of H&E stained liver sections from IL-4Rα mutant strains revealed impaired granuloma formation (Fig 4.6A) and reduced collagen production,

quantified as hydroxyproline compared to littermate control mice (Fig 4.6B). Serum AST levels were increased in IL-4Rα$^{-/-}$ mice while it was similar between CD11c^{cre}IL-4Rα$^{-/lox}$ mice and littermate control mice (Fig 4.6C). Both the CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice had significantly reduced levels of tissue IL-10 and IFN-γ compared to control mice (Fig 4.6D & E). Histological examination of liver sections showed that both CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ developed smaller granulomas around parasite eggs than the littermate control mice (Fig 4.6F). Therefore, these data suggest that the lack of IL-4/IL-13 responsive DCs ameliorates liver pathology by limiting granuloma formation and fibrosis induced by *S. mansoni* eggs.

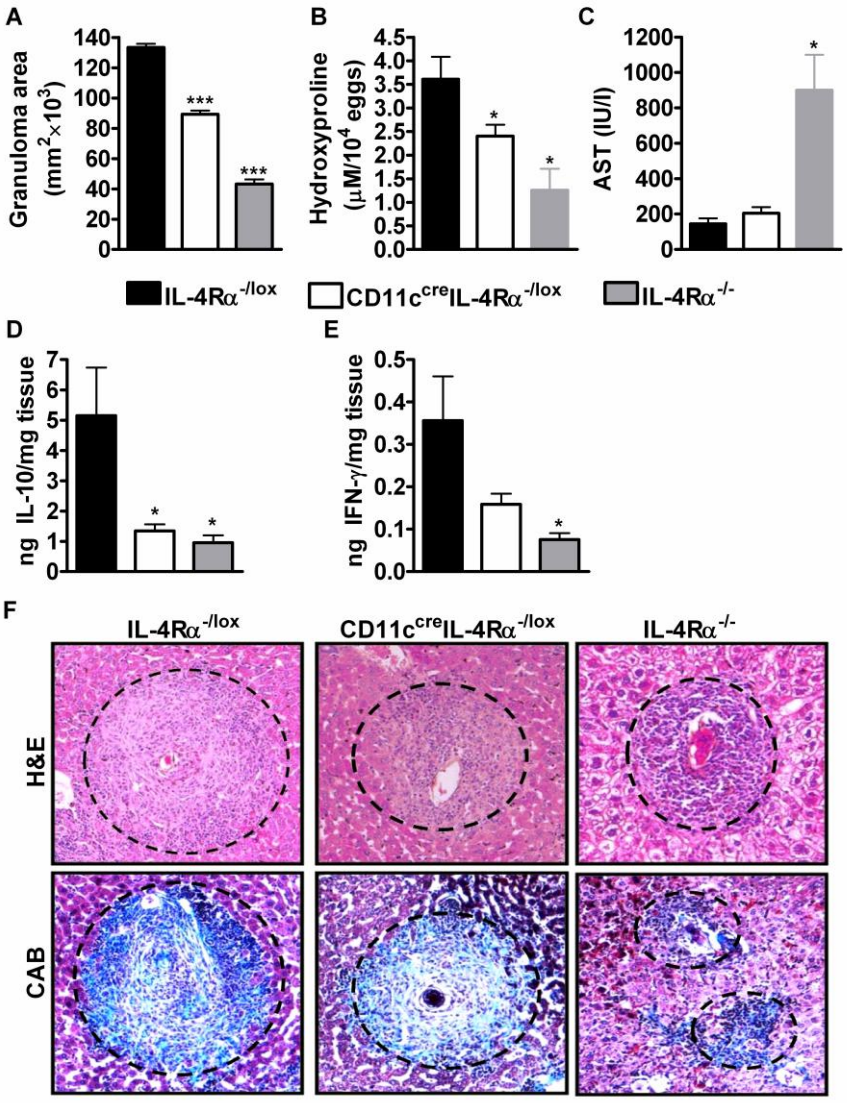

Figure 4.6: CD11c^{cre}IL-4Rα$^{-/lox}$ mice develop reduced liver pathology during acute schistosomiasis.
IL-4Rα$^{-/lox}$, CD11c^{cre}IL-4Rα$^{-/lox}$ and IL-4Rα$^{-/-}$ mice were infected with 100 *S. mansoni* cercariae and analysed 7 weeks post-infection. (**A**) Granuloma area surrounding eggs quantified by microscopic analysis on H&E stained sections. Twenty to 30 granulomas per mouse were included in the analysis. (**B**) Liver fibrosis measured as hydroxyproline normalised to egg numbers. (**C**) Hepatocellular damage measured as serum levels of aspartate transaminase enzyme. (**D**) Formalin-fixed liver sections (100×) stained with H&E for morphological analysis or CAB for collagen content. Data is representative of 2 independent experiments. n= 4-6 mice. *p< 0.05, **p< 0.01 and ***p< 0.001 vs IL-4Rα$^{-/lox}$.

To determine the contribution of IL-4/IL-13 responsive DCs to gut inflammation induced by *S. mansoni* eggs, gut hydroxyproline content and the number of *S. mansoni* eggs trapped in the tissue were quantified. This suggests that deletion of IL-4Rα signalling specifically on $CD11c^{+}$ DCs and all haematopoietic cells has no impact on worm maturity. There was no significant difference in the quantity of gut hydroxyproline or the number of eggs trapped in the tissue between mutant strains and littermate control mouse (Fig 4.7A & B). Furthermore, $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice showed reduced gut inflammation, indicated by reduced inflammatory cells surrounding the eggs, while $IL\text{-}4R\alpha^{-/-}$ mice had increased gut inflammation compared to littermate control mice (Fig 4.7C). These data suggests that IL-4/IL-13 responsive DCs may be driving immune responses mediating severe gut inflammation during *S. mansoni* infection.

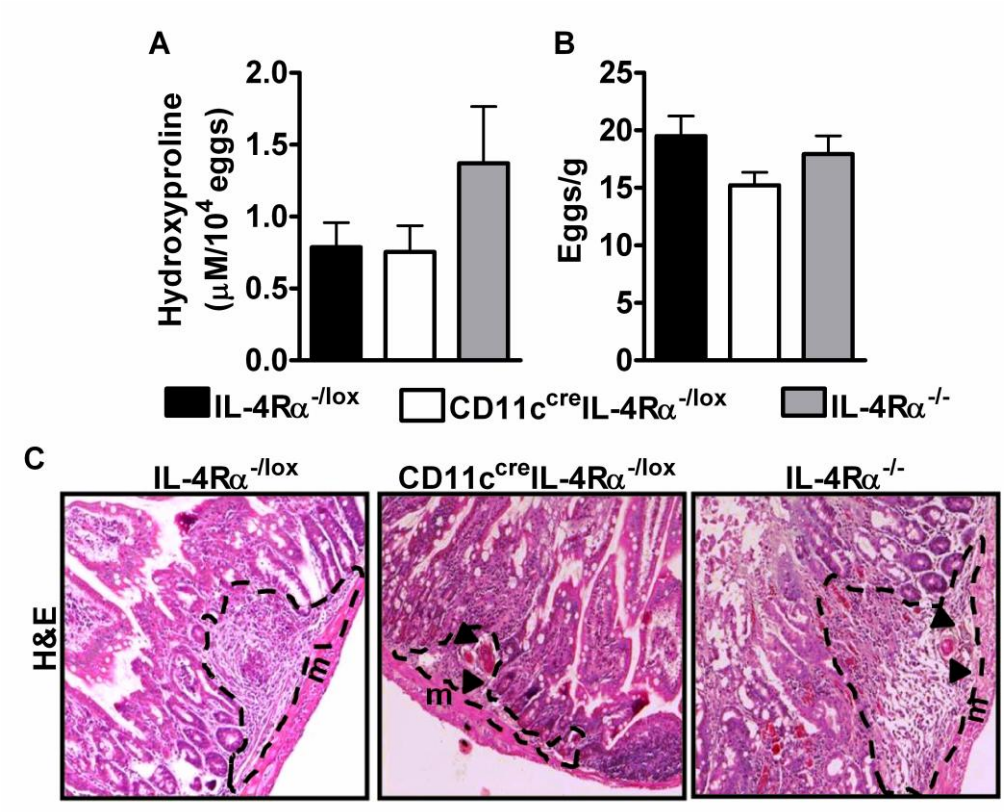

Figure 4.7: Reduced gut pathology in $CD11c^{cre}IL\text{-}4R^{-/lox}$ mice during acute schistosomiasis.
$IL\text{-}4R\alpha^{-/lox}$, $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ and $IL\text{-}4R\alpha^{-/-}$ mice were infected with 100 *S. mansoni* cercariae and analysed 7 weeks post-infection. (**A**) Intestinal fibrosis measured as hydroxyproline normalised to tissue egg numbers (mean ± SEM, n= 4-6). (**B**) Intestinal eggs numbers normalised to tissue weight (mean ± SEM, n= 4-6). (**C**) Reduced inflammation shown in H&E stained sections of the small intestine (×100). Data represents two independent experiments.

4.4 Discussion

Studies have demonstrated that IL-4 driven Th2 immune responses and granuloma formation are vital for host protection during *S. mansoni* infection [148,217,219,222]. Recent studies have delineated specific IL-4 responsive cellular populations that are crucial for prolonging survival to acute schistosomiasis. Mice deficient in IL-4/IL-13 responsive macrophages were found to be

extremely susceptible to *S. mansoni* infection due to increased hepatocellular damage, impaired egg expulsion, increased NOS-2 activity and gut inflammation that ultimately led to endotoxemia and septic shock [147]. Moreover, mice deficient in IL-4Rα expression on pan-T cells quickly succumbed to *S. mansoni* infection, despite controlling gut inflammation, in contrast to mice lacking IL-4Rα only on $CD4^+$ T cells, which resisted *S. mansoni* infection similarly to control mice [186,190]. This suggests that IL-4/IL-13 responsive non-$CD4^+$ T cells contribute to host resistance during infection [190]. However, the contribution of IL-4/IL-13 responsive $CD11c^+$ dendritic cells (DCs) in the host protection or susceptibility to *S. mansoni* infection and their impact on the development of polarised Th2 immune response and tissue pathology have not yet been fully explored. To address these critical questions, novel mice lacking IL-4Rα expression specifically on $CD11c^+$ DCs ($CD11c^{cre}$IL-$4R\alpha^{-/lox}$) were generated using the *cre*/*lox*P recombinase system under the control of the CD11c gene locus [192].

$CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice infected with *S. mansoni* developed a slow and prolonged wasting disease that ultimately resulted in 100% mortality during the chronic stages (13 weeks) of infection as compared to IL-$4R\alpha^{-/lox}$ littermate control mice that had 25% mortality throughout the infection period. In contrast, IL-$4R\alpha^{-/-}$ mice quickly succumbed to infection and developed severe cachexia immediately after the onset of egg-laying. Previous studies have demonstrated that the mechanism responsible for mortality in IL-$4R\alpha^{-/-}$ mice is caused by increased hepatocellular damage, up-regulation of proinflammatory cytokines particularly TNF-α, impaired faecal egg output, impaired granuloma formation and augmented gut inflammation and damage with subsequent septicaemia [147,148,190]. A similar mechanism was the cause of death in mice deficient in alternatively activated macrophages [147] and IL-4, IL-4/IL-13 and IL-10/IL-4 deficient mice [148,218,219,222]. Conversely, IL-10/IL-12 double deficient mice died of a distinct mechanism characterised by a wasting disease, relatively low AST levels indicating little hepatotoxicity, increased serum TNF-α levels and augmented hepatic fibrosis [222]. Infected $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice displayed low serum AST levels but increased serum TNF-α levels, suggesting that TNF-α may be contributing to mortality. High levels of IFN-γ, TNF-α and soluble TNF receptor α are associated with severe hepatosplenomegaly caused by *S. mansoni* infection in human patients [238,294]. Therefore, impairment of IL-4/IL-13 signalling on DCs is extremely detrimental to the host during the later stages of *S. mansoni* infection. Furthermore, $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ mice appear to be dying from a mechanism that is distinct from that operating in the highly susceptible IL-$4R\alpha^{-/-}$ mice.

S. mansoni eggs trapped in the host tissue induce a highly polarised Th2 immune response characterised by up-regulation of cytokines such as IL-4, IL-5 and IL-13 and type 2 anti-*Schistosoma* antibodies [217,281]. Depletion of $CD11c^+$ DCs inhibits the production of Th2 cytokines by splenocytes and $CD4^+$ T cells during synchronous challenge with *S. mansoni* eggs [286]. In addition, adoptive transfer of SEA sensitised IL-$4R\alpha^{-/-}$ BMDCs resulted in impaired production of IL-10 and IFN-γ by restimulated pLN cells, suggesting that IL-4/IL-13 responsive DCs regulate Th1 polarisation *in vivo* [287]. In our study, restimulation of total MLN cells with

SEA revealed similar production of both Th1 and Th2 cytokines in CD11^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate controls, except for diminished production of IL-13. CD11c^{cre}IL-4Rα$^{-/lox}$ mice displayed dominant type 2 antibody responses characterised by secretion of antigen-specific IgG1 and total IgE, similar to the levels produced by the IL-4Rα$^{-/lox}$ littermate control mice. In contrast, a previous study showed that partial depletion of CD11c^{+} DC impaired secretion of IgE despite unaltered levels of serum IgM and IgG1 [287]. Deletion of IL-4Rα expression on CD11c^{+} DCs had a dramatic impact on the recruitment of effector CD4^{+} T cells during infection. This agrees with previously published data, where depletion of CD11c^{+} DCs hindered induction of effector and regulatory CD4^{+} T cells during the initiation of the response against *S. mansoni* infection [286]. Abrogation of IL-4Rα expression on DCs impaired secretion of Th2 cytokines IL-5 and IL-13 and Th1 cytokine IFN-γ by CD4^{+} T cells restimulated with PMA/Ionomycin *ex vivo*, suggesting that IL-4/IL-13 responsive DCs are important for the production of cytokines by CD4^{+} T cells during infection. Likewise, secretion of Th1 and Th2 cytokines by CD4^{+} T cells was impaired following the depletion of CD11c^{+} DCs during infection in the previous study [286]. Therefore, this strongly suggests that non-CD4^{+} T cells were the source of Th1 and Th2 cytokines detected after antigen specific restimulation of total MLN cells from infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice. Beyond affecting CD4^{+} T cell responses, depletion of CD11c^{+} DCs was previously shown to inhibit B cell development by altering the proportions of marginal zone, follicular and transitional B cells [286]. Recruitment of CD19^{+} B cells was impaired in mice deficient in IL-4Rα expression on DCs and expression of activation markers MHCII and CD86 was reduced. Therefore, the absence of IL-4Rα expression on DCs seems to have a dramatic effect on the activation of both CD4^{+} T cells and CD19^{+} B cells.

In a study by Phythian-Adams and colleagues, depletion of CD11c^{+} DCs during challenge with *S. mansoni* eggs resulted in polarised production of Th1 cytokine IFN-γ and concomitantly reduced production of IL-4 and IL-10 by pLN cells stimulated with SEA [286]. In addition, production of IL-10 and IFN-γ by SEA stimulated pLN cells was found to be dependent on IL-4Rα expression on DCs [287]. However, our findings contradict this data, since the production of IL-4, IL-5, IL-10 and IFN-γ by total pLN cells stimulated with SEA was similar in CD11c^{cre}IL-4Rα$^{-/lox}$ mice compared to littermate control mice after synchronous *S. mansoni* eggs injection into the right hind footpad. In stark contrast, SEA stimulation of pLN cells from global IL-4Rα$^{-/-}$ mice resulted in dramatically impaired production of IL-4, IL-13 and IL-10, and increased production of IFN-α, suggesting a shift towards Th1 immunity. Interestingly, the lack of IL-4/IL-13 responsive DCs had a dramatic impact on the proportions of cytokine producing B cells.

Previously, *in vitro* stimulation of IL-4/IL-13 responsive BMDCs with recombinant IL-4 resulted in substantial up-regulation of mRNA transcripts and protein secretion of RELMα, Ym-1, MR and Dectin-1 [287], markers previously associated with alternatively activated macrophages [292,293]. In our study, IL-4/IL-13 unresponsive DCs were found to have unaltered expression of Ym-1 and increased iNOS expression, indicating a subtle shift towards classical activation. In addition, macrophages from CD11c^{cre}IL-4Rα$^{-/lox}$ mice displayed a classical activation phenotype

characterised by up-regulation of iNOS and down-regulation of Ym-1 expression, which could be a result of the decreased production of Th2 cytokines by $CD4^+$ T cells. Alternatively activated macrophages down-modulate organ injury caused by *S. mansoni* eggs trapped in the host tissue [147] and function as effector cells mediating host protection against *H. polygyrus* infection [295]. In contrast, alternatively activated macrophages increase susceptibility to *L. major* infection [296,297].

Mature DCs are capable of polarising Th cell immunity by influencing the cytokine microenvironment where T cell activation occurs. In the presence of pathogen products such as CpG DNA or LPS, IL-4 instructs BMDCs to up-regulate IL-12 secretion by suppressing IL-10 production [288,290,291]. Interestingly, murine DCs stimulated with SEA have a muted activation profile characterised by little or no up-regulation of maturation markers MHCII, CD80 and CD86 [298], impaired production of proinflammatory cytokines IL-12p70, TNF, IL-6 and most notably, they are unable to induce the production of anti-inflammatory cytokines IL-10 and IL-4 [299,300]. Interestingly, the production of both IL-12 and IL-10 was impaired in DCs from infected $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice after restimulation with PMA/Ionomycin *ex vivo,* supporting the previously published data. This had a dramatic impact on the production of both Th1 and Th2 cytokines by $CD4^+$ T cells from infected $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice, suggesting that IL-4/IL-13 responsive DCs may regulate Th1 and Th2 immune responses via IL-10 and IL-12 production. Accumulating reports suggest that DC-derived IL-10 is capable of promoting Th2 responses [301,302], T cell tolerance [303,304] and prevent expansion of Th1 immunity through its autocrine inhibition of IL-12 secretion [305].

Infected $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice developed smaller liver granulomas, reduced fibrosis and unaltered levels of AST, an enzyme indicating hepatocellular damage compared to littermate control mice. In accordance with previously published data, global $IL\text{-}4R\alpha^{-/-}$ mice presented impaired liver granuloma formation, reduced hepatic fibrosis and enhanced serum AST levels compared to littermate control mice [147,148,186,190]. IL-13 is known as a key cytokine mediating hepatic fibrosis by activating collagen production by fibroblasts [149,224,306] . Moreover, Th1 cytokines IL-12 and IFN-γ are capable of suppressing collagen deposition [307]. Hence, the reduced production of IL-13 by SEA restimulated MLN cells from infected $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice offers a plausible explanation for impaired hepatic fibrosis in these mice. Therefore, abrogation of IL-4/IL-13 responsiveness on DCs impairs *S. mansoni* eggs induced liver pathology that may contribute towards morbidity in the host.

Studies have unequivocally demonstrated that mice that quickly succumb to *S. mansoni* infection developed an augmented gut inflammation, accompanied by destruction of gut lumen that ultimately results in endotoxemia and septic shock [147,148,218,222]. Paradoxically, $iLck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice were able to control *S. mansoni* eggs induced gut inflammation and endotoxemia although they were highly susceptible to infection [190]. In agreement with these findings,

infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice managed to control gut inflammation as indicated by smaller granuloma size and less infiltrating cells around the eggs compared to global IL-4Rα$^{-/-}$ mice and littermate control mice. Therefore, these findings further support our contention that the mechanism responsible for mortality in CD11c^{cre}IL-4Rα$^{-/lox}$ mice is distinct from that operating in IL-4Rα$^{-/-}$ mice.

In conclusion, our study demonstrates that IL-4/IL-13 responsive DCs are crucial for conferring host protection to *S. mansoni* infection by limiting the deleterious effects of the proinflammatory cytokine TNF-α and regulating recruitment of effector CD4$^+$ T cells and CD4$^+$ T cell cytokine production. The lack of IL-4Rα expression on DCs skewed DCs and macrophage activation profiles toward classical activation as indicated by up-regulated expression of iNOS. Interestingly, the inability of DCs to respond to IL-4/IL-13 signalling resulted in impaired production of both IL-10 and IL-12, providing an explanation for diminished cytokine production by CD4$^+$ T cells.

CHAPTER 5: GENERAL DISCUSSION

General discussion

The aim of this thesis was to address the role of CD28 costimulation during recall of protective memory immune responses against secondary infection with *N. brasiliensis* and to investigate the contribution of IL-4/IL-13 responsive B cells and $CD11c^{+}$ dendritic cells in host protective immune responses during *S. mansoni* infection. In the first study, the requirement of CD28 costimulation during recall of memory responses against nematode infections was investigated using a novel mouse model ($CD28^{-/lox}Cre^{+/-}$) that allowed for inducible deletion of *cd28* gene by oral administration of tamoxifen. As expected, $CD28^{-/-}$ mice failed to expel *N. brasiliensis* adult worms during primary infection compared to C57BL/6 mice. This was further confirmed by blocking CD28 signalling with mouse anti-mouse CD28 monoclonal antibody. Previous studies have unequivocally demonstrated the importance of IL-13, IL-4Rα and STAT-6 in the expulsion of *N. brasiliensis* adult worms from infected mice [144,145,155]. The production of Th2 cytokines IL-4 and IL-13 was impaired in $CD28^{-/-}$ mice following restimulation of total lymph node cells with either NAg plus sub-optimal concentration of α-CD3 or α-CD3 alone *ex vivo*. Furthermore, humoral immunity was abrogated in $CD28^{-/-}$ mice compared to control C57BL/6 mice. Therefore, CD28 costimulation is indispensable for the development of host protective immune responses to primary infection with *N. brasiliensis*.

Importantly, mice treated with tamoxifen prior to secondary infection failed to clear *N. brasiliensis* secondary infection, similarly to the conventional $CD28^{-/-}$ mice. Both the tamoxifen treated ($CD28^{-/lox}Cre^{+/-}$+TM) and $CD28^{-/-}$ mice failed to develop sufficient Th2 immune responses indicated by reduced production of key cytokines IL-13 and IL-4 after restimulation of total MST cells with either NAg plus sub-optimal α-CD3 or α-CD3 alone *ex vivo*. Moreover, $CD28^{-/lox}Cre^{+/-}$ +TM mice exhibited impaired humoral immunity, reduced numbers of effector $CD4^{+}$ T cells and $CXCR5^{+}$ T_{FH} cells. Cognate interaction between T_{FH} cells and B cells is essential for germinal center formation and antibody production by B cells [60]. Previous studies have demonstrated that IL-4 producing Th2 cells can differentiate into T_{FH} in the reactive lymph nodes during immunisation with *S. mansoni* antigens [258] and during infection with *H. polygyrus* [259]. Together, our findings suggest that CD28 costimulation is crucial for recall of efficient memory immune responses that provide protection against *N. brasiliensis* secondary infection.

Earlier studies aimed at elucidate factors governing the mechanisms conferring host protection or susceptibility to *S. mansoni* infection utilised gene deficient mice. These studies demonstrated that

IL-4, IL-4/IL-13, IL-4Rα and STAT-6 are essential for the development of host protective Th2 immune responses and granuloma formation during *S. mansoni* infection in mice [148,149,218,219]. In our laboratory, we have generated novel mouse models lacking IL-4Rα expression on specific cell-types and these have been tested with different infectious disease models. Mice lacking IL-4Rα expression on macrophages ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$) were found to be extremely susceptible to *S. mansoni* infection due to increased production of Th1 cytokines, uncontrolled liver inflammation, impaired egg expulsion, increased NOS-2 activity and exaggerated gut pathology [147]. Therefore, this study demonstrated that IL-4/IL-13 responsive alternatively activated macrophages play an essential role in host protection during acute schistosomiasis and limit tissue injury by down-regulating egg-induced inflammation. Subsequent studies from our laboratory demonstrated the importance of IL-4Rα expression on pan-T cells [190] and smooth muscle cells [188] in the development of host protective immune responses during *S. mansoni* infection in mice. Recently, novel mouse models deficient in IL-4Rα expression on B cells [191] and $CD11c^{+}$ DCs [192] have been generated and characterised in our laboratory.

In the second study, we used the previously characterised B cell-specific IL-4Rα deficient mice ($mb1^{cre}IL\text{-}4R\alpha^{-/lox}$) [191], to investigate the role of IL-4/IL-13 responsive B cells in host protective immune response during acute schistosomiasis. In this study we used three experimental models of *S. mansoni*, acute infection model where mice were infected with 100 live cercariae, a chronic infection model where mice were infected with 30 live cercariae and a more controlled pulmonary model where mice were synchronously challenged with *S. mansoni* eggs. We found that IL-4/IL-13 responsive B cells are crucial for protection against acute schistosomiasis and down-regulate liver and gut inflammation. Macrophages from infected $mb1^{cre}IL\text{-}4R\alpha^{-/lox}$ mice displayed a classically activated phenotype characterised by up-regulation of iNOS production and reduced expression of Ym-1 compared to littermate control mice. Alternatively activated macrophages have been shown to be essential for protection against *S. mansoni* infection by limiting organ injury [147]. Importantly, production of both Th1 and Th2 cytokines by $CD19^{+}$ B cells was impaired in $mb1^{cre}IL\text{-}4R\alpha^{-/lox}$ mice compared to littermate control mice. Furthermore, the recruitment of $CXCR5^{+}$ T_{FH} cells, effector $CD4^{+}$ T cells and follicular B cells was impaired in mice deficient in IL-4Rα expression on B cells compared to littermate control mice. Together, these findings suggest that IL-4/IL-13 responsive B cells are important for development of $CD4^{+}$ T cell immunity and alternative activation of macrophages during *S. mansoni* infection.

The contribution of B cells in down-regulating granuloma formation during the chronic stages of *S. mansoni* infection is well established [148,232]. However, the requirement of IL-4/IL-13 responsive B cells in regulating granuloma formation and T cell responsiveness during the chronic stages of infection has not yet been studied. *Mb1*^{cre}IL-4Rα$^{-/lox}$ mice failed to down-regulate granuloma formation and T cell responsiveness during the chronic stages of infection. The importance of IL-4/IL-13 responsive B cells in limiting granuloma formation was further demonstrated in the pulmonary model where lungs from *mb1*^{cre}IL-4Rα$^{-/lox}$ mice had bigger granulomas compared to littermate control mice. Therefore, we have unequivocally demonstrated the importance of B cells responsive to IL-4/IL-13 signalling during down-regulation of granuloma formation induced by *S. mansoni* eggs.

It is well established that the development of a Th2 immune responses is indispensable for host protection against *S. mansoni* infection [147,148,218,219]. Hence, we hypothesised that IL-4 derived from B cells initiates the development of the host protective Th2 immune responses during *S. mansoni* infection. Our data from BM chimera mice showed that B cell derived IL-4 is not crucial for the development of sufficient Th2 immune responses during *S. mansoni* infection. The findings are in agreement with the previous findings, where IL-4 producing B cells were shown to be unnecessary for protection against *H. polygyrus* infection [90]. However, B cell derived IL-2 and TNF-α were shown to regulate immunity to *H. polygyrus* infection [90]. We suspect that a similar mechanism might operate in mice infected with *S. mansoni*. Hence, future work will involve generating BM chimeras that lack IL-2 and TNF-α production specifically by B cells. Furthermore, we plan to generate BM chimeras lacking MHC II expression on B cells to study the importance of antigen presentation by B cells and how it influences the development of host protective immune responses during *S. mansoni* infection.

In the third study, we determined the role of IL-4/IL-13 responsive CD11c^{+} DCs during *S. mansoni* infection using a novel mouse model lacking IL-4Rα expression specifically on CD11c^{+} DCs (CD11c^{cre}IL-4Rα$^{-/lox}$). In this study we used two experimental models of *S. mansoni*; acute infection model and the footpad model where *S. mansoni* eggs were injected into hind footpad of mice. Our study found that IL-4/IL-13 responsive DCs are essential for surviving *S. mansoni* infection despite controlled liver and gut inflammation. Deletion of IL-4Rα expression on DCs impaired activation of CD4^{+} T cells as indicated by abrogated intracellular production of IL-5, IL-13 and IFN-γ by CD4^{+} T cells stimulated with PMA/Ionomycin *ex vivo* and reduced numbers of

effector $CD4^+$ T cells. We further showed that the production of Th2 cytokine by total pLN cells from $CD11c^{cre}IL\text{-}4R\alpha^{-/lox}$ mice was unaltered compared to littermate control mice following restimulation with SEA. This contradicts previously published data where depletion of $CD11c^+$ DCs was shown to result in reduced production of Th2 cytokines IL-4 and IL-10 by pLN cells stimulated with SEA *ex vivo* [286]. The activation profile of DCs and macrophages showed a shift towards classical activation indicated by increased iNOS expression and a concomitant reduced expression of Arg-1, a marker for alternatively activated macrophages. Alternatively activated macrophages are associated with limiting inflammation and wound healing [292].

In conclusion, cell-specific IL-4Rα deficient mice offer a unique tool for studying the mechanisms conferring protection or susceptibility to the host during *S. mansoni* infection. The results presented in this thesis demonstrate the importance of IL-4Rα signalling on B cells and $CD11c^+$ DCs in the development of host protective immune responses to *S. mansoni* infection. The complexity of the mechanisms conferring host protective immunity to *S. mansoni* infection and the many different IL-4Rα expressing cell types involved in disease protection present significant challenges for developing an effective vaccine. Hence, more work is still needed to dissect the key factors contributing to the immunological mechanism of schistosomiasis.

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APPENDIX A

Reagents:

Anaesthetic

1.2 ml	Anaket-V (100mg/ml) (Centaur labs, Isando)
0.8 ml	Rompun (2%) (Bayer, Germany)
8.0 ml	PBS (1X)

Blocking Buffer

20g	Milk powder (spar instant) (2%)

Make up to 1L with 1X PBS

Coating Buffer

10 ml	10X PBS
8g	BSA (Merck)

Make to 1L in ddH_2O and pH 9.5

Dilution Buffer

10g	BSA (1%) (Roche)
0.2g	NaN_3 (0.02%) (Merck)

Make up to 1L with 1X PBS

FACS Buffer

0.1%	BSA (Roche)
0.05%	NaN_3 (Merck)

Make up in 1X PBS

Iscove's Modified Dulbecco's Medium (IMDM)

500 ml	IMDM (Gibco)
2.5 ml	Penicillin/streptomycin (200X)
10%	FCS

Adjust pH to 7.2 – 7.4

Phosphate Buffered Saline (PBS 10X)

80g	NaCl (1.37M)
2g	KCl (0.03M)
14.4g	H_2PO_4 (0.01M)
2.4g	KH_2PO4

Dissolve in 1L ddH_2O

Red cell lysis buffer

5 mM	EDTA	150mM	NaCl
10%	glycerol	25 mM	Tris-Cl pH 7.5
0.1%	SDS	1%	Triton-X 100
0.5%	Non idet P-40	0.5%	Deoxycholate
5 mM	PMSF		

Make to 1L with ddH_2O

Substrate Buffer

0.2g	NaN3 (0.02%)
97 ml	di-ethanolamine
0.8g	$MgCl_2.6H_2O$
700 ml	ddH_2O

Adjust the pH to 9.8 and make up to 1L with ddH_2O

Washing Buffer (20X)

20g	KCl
144g	$Na_2HPO_4.H_2O$
50 ml	Tween
20g	KH_2PO_4
800g	NaCl
100 ml	10% NaN_3

Make up to 5L with ddH_2O