THE MANY FACES OF INTERLEUKIN-4 IN HOMEOSTASIS AND DISEASE

by

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To my precious mother, Dr. Alma R. Selva, who from Heaven guides my steps.

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"Gratitude is the healthiest of all human emotions" Z. Ziglar

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LIST OF ABBREVIATIONS

SEA Schistosoma Egg Antigen

LEC Lymphatic Endothelial Cells

FDC Follicular Dendritic Cells

APC Antigen Presenting Cell

IL-4 Interleukin-4

PLN Peripheral Lymph Node

MLN Mesenteric Lymph Node

STAG Soluble Toxoplasma Gondii Antigen

BEC Blood Endothelial Cells

FRC Fibroblastic Reticular Cells

STAT-6 Signal Transducer and Activator of Transcription 6

SLO Secondary Lymphoid Organs

MIR Micro Ribonucleic acid

LTO Lymphoid Tissue Organizer

LTI Lymphoid Tissue Inducer

NFAT Nuclear Factor Of Activated T Cells

AP-1 Activator Protein 1

GATA-3 Gata Binding Protein 3

IRS Insulin Receptor Substrate

OVA Ovalbumin

NALT Nasal Associated Lymphoid Tissue

INKT Invariant Natural Killer T Cell

PBMC Peripheral Blood Mononuclear Cells

ABSTRACT

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Intensive study of interleukin-4 for more than three decades has revealed multiple functions of this cytokine in diverse processes. Nevertheless, the wide distribution of Interleukin-4 suggests the possibility of unexplored roles. Indeed, in here we present a novel role of IL-4 for the maintenance of different populations of stromal cells in peripheral lymph nodes at homeostasis and describe a role of IL-4 in the expansion of these stromal populations following antigen challenge. In consequence, IL-4 is fundamental for mounting an appropriate humoral response to a primary immunization, and absence of this cytokine is detrimental for the development of a Type 2 response. Furthermore, we describe the role of IL-4 in the immune responses of offspring antenatally exposed to *Schistosoma mansoni* antigens. Diminished IL-4 production is linked to reduced cellular T and B cells responses in offspring derived from infected mothers, which is of critical relevance to understand vaccination failure. Finally, we describe the protective role of Schistosomiasis infection in atherosclerosis and propose possible mechanism that helps explain the athero-protection. This will contribute to the discovery of novel pathways inducing protection from cardiovascular disease and help to identify possible targets for novel treatments.

CHAPTER 1. INTRODUCTION

IL-4 overview

Transcriptional regulation of IL-4

First discovered in 1982 as a B cell growth factor (Howard et al., 1982), IL-4 is now considered a pleiotropic cytokine with diverse functions in the regulation of the immune system. Among its functions, IL-4 promotes Th2 differentiation (Mosmann & Coffman, 1989), proliferation of different types of hematopoietic and non-hematopoietic populations (Yanagida et al., 1995), and it has been implicated in the development of allergic and other diseases such as atopic dermatitis and asthma (Cardoso et al., 2009), as well as in the protection of extracellular parasites such as helminths. IL-4 is produced by a wide range of immune cells such as basophils, mast cells, natural killer (NK) T cells, γδ T cells, eosinophils and CD4 T cells. The *il-4* promoter, which consists of 300 base pairs (both in human and mice) has five binding elements, known as P0 to P4, that harbor binding sites for the transcription factor, Nuclear Factor of Activated T- cells (NFAT). NFAT dephosphorylation by the phosphatase calcineurin is necessary for NFAT binding to specific sequences. Also, NFAT is essential to assemble NF-κβ p65, c-Maf, RNA polymerase II and Brahma related gene 1 (Brg1) at the regulatory site of *il-4 (Kock et al., 2014)* Moreover, the promoter region of *il-4* contains region of high affinity for Activating protein -1 (AP-1), which allows cooperative binding of NFAT and AP-1. AP-1, a heterodimer composed of basic leucine zipper (bZIP) factors such as Fos, Jun families or activating transcription factor (ATF2) and JDP subfamilies. AP-1 regulation is mostly achieved by regulation of the abundance and activity of AP-1 proteins as well as by regulation of their stability (Karin, Liu, & Zandi, 1997) In addition to AP-1 and NFAT, factors such as c-maf, STAT-6, GATA-3, Jun B, NF-kB can also regulate the

activity of the *il-4* promoter region. Nevertheless, in order to produce sufficient levels of endogenous IL-4, regulation of the distal elements in the *il-4* locus is also required. Lee GR at al. reported that GATA-3, a zinc finger DNA binding protein, regulates enhancer elements such as the intergenic DNase I hypersensitive site (HSS) and the intronic enhancer (IE) to activate IL-4 expression and thus, it functions as locus control region (LCR). (G. R. Lee, Fields, & Flavell, 2001).

Downstream, the IL-4 signaling pathway is dependent of the activation of Signal Transducer and Activator of Transcription (STAT) 6. Upon binding of IL-4 to the IL-4R, STAT-6 molecules are tyrosine phosphorylated, dimerize and translocate to the nucleus to start gene transcription. Alternatively, IL-4R binding leads to phosphorylation of Insulin Receptor Substrate (IRS)3, which recruits the tyrosine phosphatase PTP2, the adapter protein Grb2, among others to cause downstream effects (White, 1996).

IL-4 Receptor

The IL-4 Receptor (IL-4R) comprises two different kinds of receptors: Type I and Type II. Type I consists of IL-4Rα and the common gamma chain subunit of the IL-2R. It is expressed by most hematopoietic and endothelial populations, and it has been reported to be expressed in the brain (Ul-Haq, Naz, & Mesaik, 2016). Meanwhile, the type II receptor is comprised of IL-4Rα and IL-13Rα1, which can bind IL-4 and IL-13, which constitutes the reason both cytokines have overlapping biological functions. IL-4Rα, also known as CD124, is broadly expressed in hematopoietic and non-hemopoietic cells (Park, Friend, Grabstein, & Urdal, 1987) and is a member of the hematopoietin receptor superfamily. On the other hand, the γc chain subunit (CD132) is a 60 kDa protein with a more restricted expression pattern, vastly expressed in hematopoietic cells but absent in other mesenchymal cells (Wery-Zennaro, Letourneur, David,

Bertoglio, & Pierre, 1999). In addition to IL-4, γc receptor is also shared by IL-2, IL-7, IL-9, IL-15, and IL-21(Overwijk & Schluns, 2009). Binding of IL-4 to the type II receptor occurs first in the IL-4Rα chain, with very high affinity (Kd = 20–300 pM) and is followed by recruitment of the IL-13Rα1. The IL-13Rα1 (CD213a1) is 65-70kDa protein that shares 76% homology in mice and humans and binds to IL-4 with low affinity and it has been associated with different functions which include parasite clearance in the intestine (Sun et al., 2016) and macrophage differentiation into an alternative activated phenotype (Dhakal et al., 2014).

Cellular sources of IL-4

Basophils

Basophils were the main producers of IL-4 in a *Nippostrongylus brasiliensis* infection, and basophils were localized in the liver and lung, and to a reduced extent, in spleen but not in the lymph nodes during infection. Importantly, IL-4 was also expressed at steady state in the liver, suggesting that in some parasitic infections, basophils are the main non-T producers of IL-4 (Min et al., 2004). Furthermore, IL-3, IL-18 and IL-33 induce basophils to produce IL-4 *in vitro.* In addition, basophil derived IL-4 was required for Th2 responses to protease allergens (papain) and ovalbumin (OVA) (Voehringer, Shinkai, & Locksley, 2004; Yoshimoto et al., 2009). Mature human basophils, when previously primed with IL-13, can produce IL-4 upon crosslinking of IgE receptor (Brunner, Heusser, & Dahinden, 1993). In addition, Mitre and collaborators showed that in human filarial infections (*Wuchereria bancrofti, Brugia malayi, Onchocerca volvulus, and Loa loa*), when PBMC (peripheral blood mononuclear cells) from infected donors and uninfected donors were incubated with BmAg (Brugia malayi antigen), the basophils from filaria-infected donors released more IL-4 in comparison to the controls, even though similar population of basophils was present in both groups. Moreover, the frequency of basophils producing IL-4

correlated with the percentages of antigen-specific histamine (Mitre, Taylor, Kubofcik, & Nutman, 2004)

Eosinophils

In an infection model of Cryptococcosis by *Cryptococcus neoformans* it was demonstrated that eosinophils were the innate cell that contributed to Th2 development, nevertheless, eosinophil derived IL-4 was not sufficient to halt fungal dissemination to the brain (Piehler et al., 2011). In humans, purified eosinophils derived from peripheral blood were able to express *il-4* mRNA transcripts and IL-4 protein following stimulation with the calcium ionophore A23187 (Bjerke et al., 1996). By employing the 4get reporter mice (il-4 allele replaced by a knock in GFP), Chen et al were able to determine that in an allergic airway inflammation model most of the GFP^+ (IL-4) in the lung were eosinophils (93% of the $CD4^-GFP^+$)(Chen et al., 2004).

T cells

T lymphocytes are a considerable source of IL-4. Le Gros et al. found that T cells isolated from the lymph node of mice that were previously injected with IgD produced IL-4 in higher quantities than control mice after stimulation with anti CD3 and IL-2 (Le Gros, Ben-Sasson, Seder, Finkelman, & Paul, 1990). IL-4 is produced by Th0 and Th2 cells in both mice and humans (Bullens, Rafiq, Kasran, Van Gool, & Ceuppens, 1999). Likewise, IL-4 drives the differentiation of Th0 to Th2 cells. In an allergic model of asthma, effector cell recruitment and goblet cell hyperplasia were dependent on T -cell derived IL-4, this was determined by employing a conditional knock-out of IL-4 in T cells in an OVA/alum induced model of allergic inflammation, when specific depletion of IL-4 in T cells led to defects in IgG1 and IgE production and migration of eosinophils to the lung (Oeser, Maxeiner, Symowski, Stassen, & Voehringer, 2015).

Group 2 innate lymphoid cells

Group 2 innate lymphoid cells or ILC2s contribute to the protection from helminth and help in the regulation of the pathogenesis of allergy, in part due to the early production of type 2 cytokines(Nausch & Mutapi, 2018). Moreover, it was reported by Doherty et al. that upon stimulation with Leukotriene D4 (LTD4), purified ILC2s in the lungs produce significant amounts of IL-4, although the functional relevance was still unclear (Doherty et al., 2013). In a *Heligmosomoides polygyrus* model ILC2s in the lamina propria were able to secrete large amounts of IL-4, which were functionally relevant for the differentiation of $CD4^+$ Th2 cells (Pelly et al., 2016).

NK T cells

NKT cells are CD1d dependent cells with roles in autoimmunity, cancer and immune tolerance (Godfrey & Kronenberg, 2004). Among NK cells, a subset defined as $CD4^-NK1.1^-$ with a specific TCR expression of Vα14 and Vβ8.2 are capable of producing IL-4 upon ligation of the TCR and independent of TCR ligation by stimulation with IL-18 (Yoshimoto et al., 2003). Increasing evidence suggests that at steady state, a population of cells defined as iNKT cell precursors can differentiate into NKT 2 cells to secrete large amounts of IL-4. In BALB/c mice, 40% of thymic iNKT expressed IL-4 (determined by using BALB/c- KN2 reporter mice, possessing huCD2 knock-in in both *il-4* alleles (Mohrs, Wakil, Killeen, Locksley, & Mohrs, 2005). Further characterization of these cells determined that most of the IL-4 producers were GATA-3, PLZF and IRF-4 positive and were defined as NKT2. *Ex vivo*, huCD2 was observed in NKT2 cell populations, and these cells were also stimulated by self-ligands (eg. Self-lipids) (Y. J. Lee, Holzapfel, Zhu, Jameson, & Hogquist, 2013; Yoshimoto, 2018)

IL-4: Friend or foe? A little bit of both?

Interleukin-4 cytokine is a key factor in promoting T cell differentiation upon T cell activation by a stimulus. IL-4 supports T cell differentiation into a T helper (Th) 2 phenotype. In addition, other cytokines like IL-1, IL-6, and IL-13 favor a Th2 phenotype while IL-12 and IFN γ favor Th1 polarization. Thus, the Th1 and Th2 dichotomy was established, with IL-4 emerging as a prototypic Th2 cytokine and INFγ as the prototypic Th1 cytokine. While Th1 cells produce IL-2, IFN γ and TNF and fight intracellular microbes, such as viruses; Th2 cells secrete IL-4, IL-5, IL-13 and have been described for their role in parasitic infections, allergy and asthma. A fine balance between both responses is necessary to maintain adequate immune responses. For example, atopic subjects (those individuals producing elevated levels of IgE in response to allergens) have been reported to have aberrant IL-4 production (Parronchi et al., 1992). Moreover, BALB/c mice, which are skewed towards a Th2 response, are susceptible to the intracellular pathogen *Leishmania major* as opposed to C57BL/6 mice, which are skewed to a Th1 response (Kamala & Nanda, 2009), but upon administration of neutralizing antibody against IL-4 to BALB/c mice, resistance to the parasite is acquired (Choi & Reiser, 1998). On the other side, suppression of the pro-inflammatory environment during pregnancy by enhancing the anti-inflammatory Th2 response by IL-4 is fundamental for successful reproductive outcomes. Experimental studies have shown that decreased placental levels of IL-4 correlate with spontaneous abortions and recurrent miscarriages (Chatterjee, Chiasson, Bounds, & Mitchell, 2014), although this effect is most likely not solely dependent on a single cytokine.

It has been proposed that IL-4 also plays a role in autoimmune encephalomyelitis protection, with IL-4 expression in the infiltrates of recovering EAE rats, suggesting that IL-4 might be involved in the downregulation of pro-inflammatory cytokines and suppression of

inflammation(Khoury, Hancock, & Weiner, 1992). In studies aimed at the understanding of the immune response to the helminth *Schistosoma mansoni*, it has become apparent that IL-4 plays a beneficial role during schistosomiasis infection, as it protects the host from death caused by hepatocellular necrosis from hepatotoxins derived from eggs (Brunet, Finkelman, Cheever, Kopf, & Pearce, 1997). Altogether, these data reveal that IL-4 has both beneficial and detrimental effects for the host in the immune system, and that a precise regulation of its transcription/expression is needed to maintain homeostasis. Moreover, as the following chapter will discuss, IL-4 deficiency leads to peripheral lymph node organization and dysregulation of humoral responses following challenge with a type 2 antigen (Chapter II), furthermore, it is suggested that maternal schistosomiasis leads to hypo responsiveness of the offspring immune system, effects that seems to be mediated by IL-4 (Chapter III).

Schistosomiasis: Requirements for Interleukin-4

Schistosomiasis is a chronic parasitic disease caused by different species of the genus Schistosoma, which include *S. mansoni*, *S japonicum*, S. *hematobium* and *S. mekongi*; it has high prevalence in the tropics including Sub-Saharan Africa, Brazil, Southeast Asia, among others (Gray, Ross, Li, & McManus, 2011). It is recognized as one of the top ten infectious disease of global importance by the World Health Organization due to its high morbidity and mortality in endemic areas (Ndlovu & Brombacher, 2014). Moreover, the concern of re-infection and resistance of the parasite to anthelminthic treatments has raised the need to find novel treatments to tackle the disease. The clinical manifestations of the disease include fever, malaise, fatigue, diarrhea at early time points, later it develops into hepatosplenic inflammation, obstructive disease and liver fibrosis (Barsoum, Esmat, & El-Baz, 2013). Schistosomiasis is characterized by an acute phase, which predominantly is biased to a Th1 phenotype, and a chronic phase, with a marked

switch to Th2 phenotype. These dichotomous responses are both responsible for the different clinical manifestations in the host, and necessary to contain the immunopathology of the infection, as studies with nude mice have shown that these mice (lacking T cells) succumb rapidly to the disease (Byram & von Lichtenberg, 1977). Moreover, the elicited response depends on the developmental stage of the parasite. In the initial 3-5 weeks following infection there is a release of IL-12 and IFN-γ. This type 1 response decreases at 5-6 weeks post-infection, where there is a concomitant increase of type 2 cytokines caused by the release of eggs by the female worm. The type 2 response is characterized by the production of IL-4, IL-5 and IL-13, and marked eosinophilia and production of Immunoglobulin E (IgE). The granulomatous response is modulated at 10-12 weeks post infection in a process that has been shown to be dependent on IL-10 signaling (Fairfax et al., 2012; Pearce & MacDonald, 2002). Interleukin 4 impairment during schistosomiasis is associated with a deficiency in granuloma formation and hepatic fibrosis. Together with IL-13, IL-4 induces the alternative activation of macrophages. Interestingly, studies using IL-4, IL-13 or IL-4/IL-13 deficient mice have shown that although both cytokines contribute to the Th2 response, in schistosomiasis, IL-13 deficient rodents had increased survival, while IL-4 deficient mice had increased mortality, suggesting specific roles of each cytokine during infection (Fallon, Richardson, McKenzie, & McKenzie, 2000). IL-4 during infection regulates macrophage alternative activation, which is required to downregulate the type 1 responses and contribute to survival to the disease. This was demonstrated by using a $LysM^{Cre}IL\text{-}4R^{-/flox}$ mouse model, which is deficient of IL-4Ralpha specifically in the monocyte/neutrophil lineage. In this model, the mice presented increased sepsis, liver and gut damage during the acute phase, and increased Th1 responses but diminished IL-4 (and also IL-5, IL-10) was reported, suggesting that in absence of Th2 responses the host is unable to prevent death from infection (Herbert et al.,

2004). Furthermore, in *S. hematobium* infection, IL-4 upregulation correlated with decreased susceptibility to reinfection by the parasite (Medhat et al., 1998), although this study did not show causation, and definition of resistant or susceptible state heavily relied on the possibilities of exposure, which inaccurately estimates the occurrence of actual infection.

Importantly, the immunogenic response to the parasite seems to be directed to the antigens derived from the eggs, which are composed by a wide variety of glycoproteins that include IPSE/alpha-1 (IL-4 inducing principle of *S. mansoni* eggs), omega-1 and kappa-5 (Haeberlein et al., 2017; Schramm et al., 2006; Schramm et al., 2009). These egg antigens are capable of inducing IL-4 (IPSE), regulatory B cells (kappa-5), activating human and mouse basophils (IPSE), and binding IgE (IPSE) to induce a strong Th2 response. Although there are confounding factors involved during natural infection, immunization studies using schistosome derived egg antigens have further revealed the importance of IL-4 and Th2 responses in orchestrating the immune response to the parasite.

Immunoregulatory effects of IL-4 in hematopoietic and non-hematopoietic cells

Effects on hematopoietic cells

Previously described as a pleiotropic cytokine, IL-4 has been shown to regulate the proliferation of hematopoietic progenitor cells, also acting in combination with other growth factors as a co-stimulant to colony forming unit-granulocyte, macrophage, erythrocyte, megakaryocyte (platelet precursor) (Kishi, Ihle, Urdal, & Ogawa, 1989; Peschel, Paul, Ohara, & Green, 1987; Rennick et al., 1987). Originally discovered as a B cell stimulatory factor, IL-4 also interacts with T cells, mast cells and macrophages (Paul, 1987; Vazquez, Catalan-Dibene, & Zlotnik, 2015). In B cells, IL-4 induces proliferation, survival, immunoglobulin isotype switch (to

IgE and IgG1 in mice and IgG4 and IgE (Lundgren et al., 1989) in humans), and induces the expression of MHC class II (major histocompatibility complex)(Thieu et al., 2007).

In macrophages, IL-4 orchestrates proliferation of monocyte derived macrophages and accumulation of resident macrophages in the liver, spleen, and lung. Jenkins et al. have shown that injection of complexed IL-4 alone can trigger macrophage expansion, shown by BrdU incorporation and Ki-67 proliferation marker expression following IL-4 complex treatment on day 0 and day 2 (Jenkins et al., 2011). Moreover, IL-4 acts directly on macrophages to induce proliferation, and this effect relies on the intrinsic expression of IL-4Ralpha by macrophages (Jenkins et al., 2013).

Macrophages are vital for recognition and uptake of bacteria, lipids, parasitic components, particles, cellular debris and more. Their phenotype is heterogenous, with classical activated (M1) and alternatively activated (M2) on either side of the spectrum. This dichotomy affects the cellular functions of macrophages as well as their transcriptional repertoire. IL-4 (and also IL-13) induces the alternative activation of macrophages and the expression of Arginase-1, macrophage mannose receptor (Mrc-1), resistin like molecules (Relm) and chitinase like molecules (Ym1/2) (Gordon, 2003; Martinez, Helming, & Gordon, 2009; Stein, Keshav, Harris, & Gordon, 1992). This phenotype confers macrophages with the ability to orchestrate critical homeostatic functions like wound healing and tissue repair through the expression of pro-fibrotic factors like fibronectin, matrix metalloproteases (MMPs), IL-1β and TGF-β (Bogdan, Paik, Vodovotz, & Nathan, 1992). IL-4 signaling also modulates fatty acid oxidation and mitochondrial biogenesis, processes dependent on STAT6 and the induction peroxisome proliferator activated receptor (PPAR), which has been related to lipid metabolism, obesity, insulin resistance and atherogenesis (Chawla, 2010). The exact function of PPAR in atherosclerosis is still not clear, with different studies suggesting

that PPAR expression in macrophages inhibited inflammatory response, therefore attributing a role in protection against inflammatory disease (C. Jiang, Ting, & Seed, 1998), while other groups have reported that PPAR expression increases the uptake of oxidized low density lipoprotein (oxLDL)(Tontonoz, Nagy, Alvarez, Thomazy, & Evans, 1998). Despite many reports, research on the role of macrophages on metabolism and athero-protection are still needed to fully understand the role of IL-4 mediated M2 macrophages on metabolic and inflammatory diseases of global concern.

Finally, AA macrophages participate in the response to allergy and helminth infections. As previously mentioned, in a rodent model of *Schistosoma mansoni*, the absence of the IL-4 pathway in macrophages led to increased mortality rates (Herbert et al., 2004). Moreover, studies utilizing Schistosoma to study the fibrotic response have reported that M2 macrophages constitute 20-30% of egg induced cellular granulomatous content in the liver (Hesse et al., 2001), which highlights the importance of these cells in granuloma regulation, although it is not yet clear the specific role or mechanism(s) used by M2 macrophages to exert their function (s).

Effects on non-hematopoietic cells

The study of mesenchymal (stromal, non-hematopoietic origin) cells has seen particular advances in the last few years, which has led to new insights into the function and regulation of these cells (Keating, 2012). Until recently, it was believed that stromal cells were only necessary to provide support to hematopoietic cells, describing them as passive cells, but now they have been identified to be involved in diverse processes that include maintenance of peripheral tolerance, tumor evasion, fetal- maternal tolerance, and promotion of T regulatory cells via IL-10 (Fayyad-Kazan, Faour, Badran, Lagneaux, & Najar, 2016; Mattar & Bieback, 2015; Nauta & Fibbe, 2007; Zhao et al., 2010). It has become clear that mesenchymal cells interact closely with immune cells

and provide cues to maintain the homeostasis of microenvironments. Likewise, these cells are also regulated by molecules secreted by immune cells. For example, non-hematopoietic cells express the type I IL-4 receptor, suggesting that they are modulated by IL-4.

Vicetti Miguel et al. reported that eosinophil-derived IL-4 promotes the expansion of a population of endometrial stromal cell *ex vivo* in a mouse model of genital *Chlamydia trachomatis* infection, with this dependence mediated by IL-4Ra. Interestingly, mice that had defects in the IL-4 pathway had more *Chlamydia* induced endometrial damage compared to wild type mice (Vicetti Miguel et al., 2017), suggesting a role of this cytokine in the regulation of disease. I*n vitro* experiments geared to identifying the role of IL-4 in endometriosis have revealed that IL-4 stimulates the phosphorylation of p38MAPK, SAPK/JNK, and p42/44 MAPK in endometrial stromal cells (ESC), and that treatment of ESC with different concentrations of IL-4 significantly increased ESC cell number. This effect was counteracted by the addition of neutralizing IL-4 receptor (OuYang et al., 2008).

In a model of follicular lymphoma (FL), IL-4 modulated the properties of infiltrating stromal cells by increasing the secretion of CXCL12 (also known as stromal cell derived factor 1, fundamental in induction of migration of hematopoietic progenitors and stem cells by interacting with CXCR4 (Janssens, Struyf, & Proost, 2018)) in mesenchymal precursors. CXCL12 induced malignant B cell infiltration and adhesion. Overall, IL-4 preferentially induced a VCAM-1^{hi}podoplanin^{lo}CXCL12^{hi} phenotype, suggesting that IL-4 indeed contributes to the phenotype of follicular lymphoma and, as result it could be useful as a target for treatment of FL (Pandey et al., 2017). Finally, in fibroblasts, IL-4 has been shown to induce type I and type III collagen, fibronectin and glycosaminoglycans secretion as well as ICAM-1 expression (Doucet et al., 1998), molecules that are essential for the homeostatic role of these cells.

Regulation and development of lymphoid organs

Secondary lymphoid organs

Secondary lymphoid organs (SLO) orchestrate the adaptive immune response to foreign antigens, constituting the site where specialized immune cells are primed by antigen encounter (Randall, Carragher, & Rangel-Moreno, 2008). Secondary lymphoid organs include lymph nodes, which are strategically distributed in the body, the spleen, Peyer's patches, tonsils and nasal associated lymphoid tissue (NALT) (Fu & Chaplin, 1999). Each organ possesses a characteristic organization with some features shared amongst all SLOs. In general, all SLOs possess T and B cells that are packaged in a differentiated compartment that fulfills specific functions. SLO development occurs during embryogenesis or in the early postnatal period. Despite shared lymphocyte organization, specific molecular cues and cellular interactions are required in each tissue. For example, lymph nodes (LN) require the interaction of lymphoid tissue inducer (LTi) cells and lymphoid tissue organizer (LTo) during developmental formation. In peripheral lymph node and mesenteric lymph node two different organizer populations have been identified (Cupedo et al., 2004), which suggest a differential signal requirement. In the early stages of the LN formation, the anlagen is surrounded by endothelial cells and blood vascular endothelial cell that form the primitive lymph node and serve as precursors of differentiated cells in the LN (Blum & Pabst, 2006). In mice, specification of lymphatic endothelial cells take place at approximately embryonic day 10 (E10), this process is followed by the formation of the initial lymphatic structures (Benezech et al., 2010; van de Pavert & Mebius, 2014). In humans, the initiation of lymph node formation is similar to mice, with the assembly of endothelial cells forming a lymph sac at around 8-11 weeks of gestation (Sabin, 1916).

At approximately E15, the lymphotoxin from LTi cell clusters ligates the lymphotoxin receptor (LTβR) from LTo, which in turn induces the upregulation of adhesion molecules and chemokines such as CXCL13, known as a strong chemoattractant for B cells. (Ansel & Cyster, 2001). Besides B cells, $CD3^{-}$ $CD4^{+}$ IL-7Rα^{hi} that express CXCR5 are also recruited. (Ohl et al., 2003). In a positive feedback loop mechanism, CXCL13 expression induces lymphotoxin, which is key to lymphoid formation as shown by studies using LTα, LTβ and LTβR deficient mice. These mice exhibited serious defects in lymphoid organogenesis, demonstrated by almost complete lack of Peyer's patches and by the presence of only rudimentary lymph nodes (Banks et al., 1995; Futterer, Mink, Luz, Kosco-Vilbois, & Pfeffer, 1998; Koni et al., 1997). These studies revealed the fundamental role of lymphotoxin in lymphoid organogenesis during the first stages of development, since its absence inhibited lymph node development, and even after lymphotoxin was restored, the formation of LN could not be re-established.

In the next stages of development, lymphocytes start to flood into the lymph nodes, stimulated by chemokine expression and the precursors of mesenchymal populations such as follicular dendritic cells (FDC) downregulate cell specific markers. Although it is not clear how cells differentiate from precursors cells to differentiated stromal cells, efforts in identifying the origins of FDCs are underway. Recently, it was suggested that FDC arise from cells that share similarities with those located in the walls of blood vessels, identical to mural cells that express the marker platelet derived growth factor receptor β (PDGFR). It is reasoned that FDC do no express this marker due to loss during maturation, although specific timepoints and mechanism remain unclear (Krautler et al., 2012).

Kinetic studies of cells in the lymph node have found the first fibroblastic reticulum cells at around 16 weeks of gestation in humans, highlighting the formation of a T cell zone. Similarly,

demarcated B cell follicles with closely related CD21 and CD35 FDC are observed (Cupedo, 2011; Markgraf, von Gaudecker, & Muller-Hermelink, 1982; Westerga & Timens, 1989). In both mice and humans, the developed lymph node is characterized by a combination of populations of hematopoietic origins (lymphocytes) and stromal cells. The T cells are in close contact with fibroblastic reticular cells (FRC) that provide scaffold for lymphocyte migration (Roozendaal et al., 2009). FRC also secrete chemokines such as CCL19 and CCL21 that help in the regulation of interaction of T cell and dendritic cells (Siegert & Luther, 2012), while FDC interact with B cells and retain antigen (Kranich & Krautler, 2016).

Interestingly, in a model of helminth infection, IL-4 induced remodeling of FRC in the mesenteric lymph node and *de novo* formation of germinal centers by the IL-4Rα signaling pathway. In addition, IL-4Rα signaling was required for lymphotoxin expression. (Dubey et al., 2016). Similarly, a function for IL-4 in the regulation of lymphotoxin was postulated by Gramaglia et al., who reported that IL-4 downregulated LTαβ expression in effector T cells (Gramaglia, Mauri, Miner, Ware, & Croft, 1999). Nevertheless, it remains unclear whether this effect was direct and what are other physiological repercussions this finding might have on immune regulation.

IL-4 in pregnancy and during fetal development

Diverse cytokines have been associated to different roles in early stages of pregnancy including during fetal adhesion, implantation and placental growth (Schafer-Somi, 2003). In humans, the immunologic recognition of pregnancy is necessary for successful outcomes, and since decades ago researchers have postulated pregnancy to be a Th2 phenomenon, highlighting the importance of anti-inflammatory cytokines and immune recognition in the reproductive process (Wegmann, Lin, Guilbert, & Mosmann, 1993). Furthermore, this Th2 bias is thought to be of relevance to avoid rejection of half-foreign antigens from the fetus. On the contrary, several

pro-inflammatory cytokines including TNF, IFNγ and TNF-related apoptosis-inducing ligand (TRAIL) are considered harmful for embryogenesis, implantation and development when encountered in high concentrations (Hill, Haimovici, & Anderson, 1987; Riley, Heeley, Wyman, Schlichting, & Moley, 2004) The Th2 bias during pregnancy has been suggested to be induced by factors originating from the fertilized ovum (Kelemen, Paldi, Tinneberg, Torok, & Szekeres-Bartho, 1998). Interestingly, there seems to be other factors sustaining the Th2 environment, since Th2 cytokines (IL-4, IL-5 and IL-10) were detected throughout the three trimesters of pregnancy (Riley et al., 2004). During this process, IL-4 production was linked, but not limited, to immune cells, since decidua, amniochorionic membranes, cytotrophoblasts, and both maternal and fetal endothelial cells were also implicated in its production (Jones, Finlay-Jones, & Hart, 1997; Riley et al., 2004). Surprisingly, IL-10 and IL-4 double knockout mice were able to complete allogeneic pregnancies, as long as the mice were maintained under clean conditions (Svensson, Arvola, Sallstrom, Holmdahl, & Mattsson, 2001). Chatterjee and collaborators determined that anti-inflammatory cytokines like IL-4 are required to prevent hypertension during pregnancy, as pregnant IL-4 deficient mice presented increased proteinuria and endothelial dysfunction in comparison to controls (Chatterjee et al., 2013).Moreover, injection of IL-4 in the mouse model of spontaneous abortion (CBA/J×DBA/2) decreased the rate of embryo resorption in comparison to untreated mice (P. J. Jiang, Zhao, Bao, Xiao, & Xiong, 2009) In addition to this, clinical reports point to decreased levels of IL-4 (and other type 2 cytokines) in women with recurrent miscarriages and reproductive failures (Hanzlikova, Ulcova-Gallova, Malkusova, Sefrna, & Panzner, 2009; Piccinni et al., 1998)In neonates, lack of IL-4 alone did not lead to loss of immune tolerance, but IL-13 and IL-4 were necessary to induce immune tolerance to foreign antigens (Inoue, Konieczny, Wagener, McKenzie, & Lakkis, 2001). Altogether, there is still a considerable

gap in knowledge regarding the factors and cells involved in each steps of pregnancy, the understanding of the contribution of determined hormones to the regulation of the immune microenvironment during pregnancy is not well understood, although it has been speculated that progesterone might be a contributor of a switch from a Th1 microenvironment to a Th2.

References

Ansel, K. M., & Cyster, J. G. (2001). Chemokines in lymphopoiesis and lymphoid organ development. *Curr Opin Immunol, 13*(2), 172-179.

Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., . . . Mucenski, M. L. (1995). Lymphotoxin-alpha-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. *J Immunol, 155*(4), 1685-1693.

Barsoum, R. S., Esmat, G., & El-Baz, T. (2013). Human schistosomiasis: clinical perspective: review. *J Adv Res, 4*(5), 433-444. doi:10.1016/j.jare.2013.01.005

Benezech, C., White, A., Mader, E., Serre, K., Parnell, S., Pfeffer, K., . . . Caamano, J. H. (2010). Ontogeny of stromal organizer cells during lymph node development. *J Immunol, 184*(8), 4521-4530. doi:10.4049/jimmunol.0903113

Bjerke, T., Gaustadnes, M., Nielsen, S., Nielsen, L. P., Schiotz, P. O., Rudiger, N., . . . Poulsen, L. K. (1996). Human blood eosinophils produce and secrete interleukin 4. *Respir Med, 90*(5), 271-277.

Blum, K. S., & Pabst, R. (2006). Keystones in lymph node development. *J Anat, 209*(5), 585-595. doi:10.1111/j.1469-7580.2006.00650.x

Bogdan, C., Paik, J., Vodovotz, Y., & Nathan, C. (1992). Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. *J Biol Chem, 267*(32), 23301-23308.

Brunet, L. R., Finkelman, F. D., Cheever, A. W., Kopf, M. A., & Pearce, E. J. (1997). IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. *J Immunol, 159*(2), 777-785.

Brunner, T., Heusser, C. H., & Dahinden, C. A. (1993). Human peripheral blood basophils primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. *J Exp Med, 177*(3), 605-611.

Bullens, D. M., Rafiq, K., Kasran, A., Van Gool, S. W., & Ceuppens, J. L. (1999). Naive human T cells can be a source of IL-4 during primary immune responses. *Clin Exp Immunol, 118*(3), 384-391.

Byram, J. E., & von Lichtenberg, F. (1977). Altered schistosome granuloma formation in nude mice. *Am J Trop Med Hyg, 26*(5 Pt 1), 944-956.

Cardoso, C. R., Provinciatto, P. R., Godoi, D. F., Ferreira, B. R., Teixeira, G., Rossi, M. A., . . . Silva, J. S. (2009). IL-4 regulates susceptibility to intestinal inflammation in murine food allergy. *Am J Physiol Gastrointest Liver Physiol, 296*(3), G593-600. doi:10.1152/ajpgi.90431.2008

Chatterjee, P., Chiasson, V. L., Bounds, K. R., & Mitchell, B. M. (2014). Regulation of the Anti-Inflammatory Cytokines Interleukin-4 and Interleukin-10 during Pregnancy. *Front Immunol, 5*, 253. doi:10.3389/fimmu.2014.00253

Chatterjee, P., Kopriva, S. E., Chiasson, V. L., Young, K. J., Tobin, R. P., Newell-Rogers, K., & Mitchell, B. M. (2013). Interleukin-4 deficiency induces mild preeclampsia in mice. *J Hypertens, 31*(7), 1414-1423; discussion 1423. doi:10.1097/HJH.0b013e328360ae6c

Chawla, A. (2010). Control of macrophage activation and function by PPARs. *Circ Res, 106*(10), 1559-1569. doi:10.1161/CIRCRESAHA.110.216523

Chen, L., Grabowski, K. A., Xin, J. P., Coleman, J., Huang, Z., Espiritu, B., . . . Huang, H. (2004). IL-4 induces differentiation and expansion of Th2 cytokine-producing eosinophils. *J Immunol, 172*(4), 2059-2066.

Choi, P., & Reiser, H. (1998). IL-4: role in disease and regulation of production. *Clin Exp Immunol, 113*(3), 317-319.

Cupedo, T. (2011). Human lymph node development: An inflammatory interaction. *Immunol Lett, 138*(1), 4-6. doi:10.1016/j.imlet.2011.02.008

Cupedo, T., Vondenhoff, M. F., Heeregrave, E. J., De Weerd, A. E., Jansen, W., Jackson, D. G., . . . Mebius, R. E. (2004). Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. *J Immunol, 173*(5), 2968-2975.

Dhakal, M., Hardaway, J. C., Guloglu, F. B., Miller, M. M., Hoeman, C. M., Zaghouani, A. A., . . . Zaghouani, H. (2014). IL-13Ralpha1 is a surface marker for M2 macrophages influencing their differentiation and function. *Eur J Immunol, 44*(3), 842-855. doi:10.1002/eji.201343755

Doherty, T. A., Khorram, N., Lund, S., Mehta, A. K., Croft, M., & Broide, D. H. (2013). Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol, 132*(1), 205-213. doi:10.1016/j.jaci.2013.03.048

Doucet, C., Brouty-Boye, D., Pottin-Clemenceau, C., Canonica, G. W., Jasmin, C., & Azzarone, B. (1998). Interleukin (IL) 4 and IL-13 act on human lung fibroblasts. Implication in asthma. *J Clin Invest, 101*(10), 2129-2139. doi:10.1172/JCI741

Dubey, L. K., Lebon, L., Mosconi, I., Yang, C. Y., Scandella, E., Ludewig, B., . . . Harris, N. L. (2016). Lymphotoxin-Dependent B Cell-FRC Crosstalk Promotes De Novo Follicle Formation and Antibody Production following Intestinal Helminth Infection. *Cell Rep, 15*(7), 1527-1541. doi:10.1016/j.celrep.2016.04.023

Fairfax, K. C., Amiel, E., King, I. L., Freitas, T. C., Mohrs, M., & Pearce, E. J. (2012). IL-10R blockade during chronic schistosomiasis mansoni results in the loss of B cells from the liver and the development of severe pulmonary disease. *PLoS Pathog, 8*(1), e1002490. doi:10.1371/journal.ppat.1002490

Fallon, P. G., Richardson, E. J., McKenzie, G. J., & McKenzie, A. N. (2000). Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. *J Immunol, 164*(5), 2585-2591.

Fayyad-Kazan, H., Faour, W. H., Badran, B., Lagneaux, L., & Najar, M. (2016). The immunomodulatory properties of human bone marrow-derived mesenchymal stromal cells are defined according to multiple immunobiological criteria. *Inflamm Res, 65*(6), 501-510. doi:10.1007/s00011-016-0933-2

Fu, Y. X., & Chaplin, D. D. (1999). Development and maturation of secondary lymphoid tissues. *Annu Rev Immunol, 17*, 399-433. doi:10.1146/annurev.immunol.17.1.399

Futterer, A., Mink, K., Luz, A., Kosco-Vilbois, M. H., & Pfeffer, K. (1998). The lymphotoxin beta receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity, 9*(1), 59-70.

Godfrey, D. I., & Kronenberg, M. (2004). Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest, 114*(10), 1379-1388. doi:10.1172/JCI23594

Gordon, S. (2003). Alternative activation of macrophages. *Nat Rev Immunol, 3*(1), 23-35. doi:10.1038/nri978

Gramaglia, I., Mauri, D. N., Miner, K. T., Ware, C. F., & Croft, M. (1999). Lymphotoxin alphabeta is expressed on recently activated naive and Th1-like CD4 cells but is down-regulated by IL-4 during Th2 differentiation. *J Immunol, 162*(3), 1333-1338.

Gray, D. J., Ross, A. G., Li, Y. S., & McManus, D. P. (2011). Diagnosis and management of schistosomiasis. *BMJ, 342*, d2651. doi:10.1136/bmj.d2651

Haeberlein, S., Obieglo, K., Ozir-Fazalalikhan, A., Chaye, M. A. M., Veninga, H., van der Vlugt, L., . . . Smits, H. H. (2017). Schistosome egg antigens, including the glycoprotein IPSE/alpha-1, trigger the development of regulatory B cells. *PLoS Pathog, 13*(7), e1006539. doi:10.1371/journal.ppat.1006539

Hanzlikova, J., Ulcova-Gallova, Z., Malkusova, I., Sefrna, F., & Panzner, P. (2009). TH1-TH2 response and the atopy risk in patients with reproduction failure. *Am J Reprod Immunol, 61*(3), 213-220. doi:10.1111/j.1600-0897.2009.00683.x

Herbert, D. R., Holscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., . . . Brombacher, F. (2004). Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity, 20*(5), 623-635.

Hesse, M., Modolell, M., La Flamme, A. C., Schito, M., Fuentes, J. M., Cheever, A. W., . . . Wynn, T. A. (2001). Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol, 167*(11), 6533-6544.

Hill, J. A., Haimovici, F., & Anderson, D. J. (1987). Products of activated lymphocytes and macrophages inhibit mouse embryo development in vitro. *J Immunol, 139*(7), 2250-2254.

Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hamaoka, T., & Paul, W. E. (1982). Identification of a T cell-derived b cell growth factor distinct from interleukin 2. *J Exp Med, 155*(3), 914-923.

Inoue, Y., Konieczny, B. T., Wagener, M. E., McKenzie, A. N., & Lakkis, F. G. (2001). Failure to induce neonatal tolerance in mice that lack both IL-4 and IL-13 but not in those that lack IL-4 alone. *J Immunol, 167*(2), 1125-1128.

Janssens, R., Struyf, S., & Proost, P. (2018). The unique structural and functional features of CXCL12. *Cell Mol Immunol, 15*(4), 299-311. doi:10.1038/cmi.2017.107

Jenkins, S. J., Ruckerl, D., Cook, P. C., Jones, L. H., Finkelman, F. D., van Rooijen, N., . . . Allen, J. E. (2011). Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation. *Science, 332*(6035), 1284-1288. doi:10.1126/science.1204351

Jenkins, S. J., Ruckerl, D., Thomas, G. D., Hewitson, J. P., Duncan, S., Brombacher, F., . . . Allen, J. E. (2013). IL-4 directly signals tissue-resident macrophages to proliferate beyond homeostatic levels controlled by CSF-1. *J Exp Med, 210*(11), 2477-2491. doi:10.1084/jem.20121999

Jiang, C., Ting, A. T., & Seed, B. (1998). PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature, 391*(6662), 82-86. doi:10.1038/34184

Jiang, P. J., Zhao, A. M., Bao, S. M., Xiao, S. J., & Xiong, M. (2009). Expression of chemokine receptors CCR3, CCR5 and CXCR3 on CD4(+) T cells in CBA/JxDBA/2 mouse model, selectively induced by IL-4 and IL-10, regulates the embryo resorption rate. *Chin Med J (Engl), 122*(16), 1917-1921.

Jones, C. A., Finlay-Jones, J. J., & Hart, P. H. (1997). Type-1 and type-2 cytokines in human late-gestation decidual tissue. *Biol Reprod, 57*(2), 303-311.

Kamala, T., & Nanda, N. K. (2009). Protective response to Leishmania major in BALB/c mice requires antigen processing in the absence of DM. *J Immunol, 182*(8), 4882-4890. doi:10.4049/jimmunol.0803956

Karin, M., Liu, Z., & Zandi, E. (1997). AP-1 function and regulation. *Curr Opin Cell Biol, 9*(2), 240-246.

Keating, A. (2012). Mesenchymal stromal cells: new directions. *Cell Stem Cell, 10*(6), 709-716. doi:10.1016/j.stem.2012.05.015

Kelemen, K., Paldi, A., Tinneberg, H., Torok, A., & Szekeres-Bartho, J. (1998). Early recognition of pregnancy by the maternal immune system. *Am J Reprod Immunol, 39*(6), 351-355.

Khoury, S. J., Hancock, W. W., & Weiner, H. L. (1992). Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor beta, interleukin 4, and prostaglandin E expression in the brain. *J Exp Med, 176*(5), 1355-1364.

Kishi, K., Ihle, J. N., Urdal, D. L., & Ogawa, M. (1989). Murine B-cell stimulatory factor-1 (BSF-1)/interleukin-4 (IL-4) is a multilineage colony-stimulating factor that acts directly on primitive hemopoietic progenitors. *J Cell Physiol, 139*(3), 463-468. doi:10.1002/jcp.1041390303

Kock, J., Kreher, S., Lehmann, K., Riedel, R., Bardua, M., Lischke, T., . . . Chang, H. D. (2014). Nuclear factor of activated T cells regulates the expression of interleukin-4 in Th2 cells in an all-or-none fashion. *J Biol Chem, 289*(39), 26752-26761. doi:10.1074/jbc.M114.587865

Koni, P. A., Sacca, R., Lawton, P., Browning, J. L., Ruddle, N. H., & Flavell, R. A. (1997). Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity, 6*(4), 491-500.

Kranich, J., & Krautler, N. J. (2016). How Follicular Dendritic Cells Shape the B-Cell Antigenome. *Front Immunol, 7*, 225. doi:10.3389/fimmu.2016.00225

Krautler, N. J., Kana, V., Kranich, J., Tian, Y., Perera, D., Lemm, D., . . . Aguzzi, A. (2012). Follicular dendritic cells emerge from ubiquitous perivascular precursors. *Cell, 150*(1), 194-206. doi:10.1016/j.cell.2012.05.032

Le Gros, G., Ben-Sasson, S. Z., Seder, R., Finkelman, F. D., & Paul, W. E. (1990). Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Exp Med, 172*(3), 921-929.

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

Lee, Y. J., Holzapfel, K. L., Zhu, J., Jameson, S. C., & Hogquist, K. A. (2013). Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol, 14*(11), 1146-1154. doi:10.1038/ni.2731

Lundgren, M., Persson, U., Larsson, P., Magnusson, C., Smith, C. I., Hammarstrom, L., & Severinson, E. (1989). Interleukin 4 induces synthesis of IgE and IgG4 in human B cells. *Eur J Immunol, 19*(7), 1311-1315. doi:10.1002/eji.1830190724

Markgraf, R., von Gaudecker, B., & Muller-Hermelink, H. K. (1982). The development of the human lymph node. *Cell Tissue Res, 225*(2), 387-413.

Martinez, F. O., Helming, L., & Gordon, S. (2009). Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol, 27*, 451-483. doi:10.1146/annurev.immunol.021908.132532

Mattar, P., & Bieback, K. (2015). Comparing the Immunomodulatory Properties of Bone Marrow, Adipose Tissue, and Birth-Associated Tissue Mesenchymal Stromal Cells. *Front Immunol, 6*, 560. doi:10.3389/fimmu.2015.00560

Medhat, A., Shehata, M., Bucci, K., Mohamed, S., Dief, A. D., Badary, S., . . . King, C. L. (1998). Increased interleukin-4 and interleukin-5 production in response to Schistosoma haematobium adult worm antigens correlates with lack of reinfection after treatment. *J Infect Dis, 178*(2), 512-519.

Min, B., Prout, M., Hu-Li, J., Zhu, J., Jankovic, D., Morgan, E. S., . . . Paul, W. E. (2004). Basophils produce IL-4 and accumulate in tissues after infection with a Th2-inducing parasite. *J Exp Med, 200*(4), 507-517. doi:10.1084/jem.20040590

Mitre, E., Taylor, R. T., Kubofcik, J., & Nutman, T. B. (2004). Parasite antigen-driven basophils are a major source of IL-4 in human filarial infections. *J Immunol, 172*(4), 2439-2445.

Mohrs, K., Wakil, A. E., Killeen, N., Locksley, R. M., & Mohrs, M. (2005). A two-step process for cytokine production revealed by IL-4 dual-reporter mice. *Immunity, 23*(4), 419-429. doi:10.1016/j.immuni.2005.09.006

Mosmann, T. R., & Coffman, R. L. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol, 7*, 145-173. doi:10.1146/annurev.iy.07.040189.001045

Nausch, N., & Mutapi, F. (2018). Group 2 ILCs: A way of enhancing immune protection against human helminths? *Parasite Immunol, 40*(2). doi:10.1111/pim.12450

Nauta, A. J., & Fibbe, W. E. (2007). Immunomodulatory properties of mesenchymal stromal cells. *Blood, 110*(10), 3499-3506. doi:10.1182/blood-2007-02-069716

Ndlovu, H., & Brombacher, F. (2014). Role of IL-4Ralpha during acute schistosomiasis in mice. *Parasite Immunol, 36*(9), 421-427. doi:10.1111/pim.12080

Oeser, K., Maxeiner, J., Symowski, C., Stassen, M., & Voehringer, D. (2015). T cells are the critical source of IL-4/IL-13 in a mouse model of allergic asthma. *Allergy, 70*(11), 1440-1449. doi:10.1111/all.12705

Ohl, L., Henning, G., Krautwald, S., Lipp, M., Hardtke, S., Bernhardt, G., . . . Forster, R. (2003). Cooperating mechanisms of CXCR5 and CCR7 in development and organization of secondary lymphoid organs. *J Exp Med, 197*(9), 1199-1204. doi:10.1084/jem.20030169

OuYang, Z., Hirota, Y., Osuga, Y., Hamasaki, K., Hasegawa, A., Tajima, T., . . . Taketani, Y. (2008). Interleukin-4 stimulates proliferation of endometriotic stromal cells. *Am J Pathol, 173*(2), 463-469. doi:10.2353/ajpath.2008.071044

Overwijk, W. W., & Schluns, K. S. (2009). Functions of gammaC cytokines in immune homeostasis: current and potential clinical applications. *Clin Immunol, 132*(2), 153-165. doi:10.1016/j.clim.2009.03.512

Pandey, S., Mourcin, F., Marchand, T., Nayar, S., Guirriec, M., Pangault, C., . . . Tarte, K. (2017). IL-4/CXCL12 loop is a key regulator of lymphoid stroma function in follicular lymphoma. *Blood, 129*(18), 2507-2518. doi:10.1182/blood-2016-08-737239

Park, L. S., Friend, D., Grabstein, K., & Urdal, D. L. (1987). Characterization of the high-affinity cell-surface receptor for murine B-cell-stimulating factor 1. *Proc Natl Acad Sci U S A, 84*(6), 1669-1673.

Parronchi, P., De Carli, M., Manetti, R., Simonelli, C., Piccinni, M. P., Macchia, D., . . . Romagnani, S. (1992). Aberrant interleukin (IL)-4 and IL-5 production in vitro by CD4+ helper T cells from atopic subjects. *Eur J Immunol, 22*(6), 1615-1620. doi:10.1002/eji.1830220640

Paul, W. E. (1987). Interleukin 4/B cell stimulatory factor 1: one lymphokine, many functions. *FASEB J, 1*(6), 456-461.

Pearce, E. J., & MacDonald, A. S. (2002). The immunobiology of schistosomiasis. *Nat Rev Immunol, 2*(7), 499-511. doi:10.1038/nri843

Pelly, V. S., Kannan, Y., Coomes, S. M., Entwistle, L. J., Ruckerl, D., Seddon, B., . . . Wilson, M. S. (2016). IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection. *Mucosal Immunol, 9*(6), 1407-1417. doi:10.1038/mi.2016.4

Peschel, C., Paul, W. E., Ohara, J., & Green, I. (1987). Effects of B cell stimulatory factor-1/interleukin 4 on hematopoietic progenitor cells. *Blood, 70*(1), 254-263.

Piccinni, M. P., Beloni, L., Livi, C., Maggi, E., Scarselli, G., & Romagnani, S. (1998). Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. *Nat Med, 4*(9), 1020-1024. doi:10.1038/2006

Piehler, D., Stenzel, W., Grahnert, A., Held, J., Richter, L., Kohler, G., . . . Muller, U. (2011). Eosinophils contribute to IL-4 production and shape the T-helper cytokine profile and inflammatory response in pulmonary cryptococcosis. *Am J Pathol, 179*(2), 733-744. doi:10.1016/j.ajpath.2011.04.025

Randall, T. D., Carragher, D. M., & Rangel-Moreno, J. (2008). Development of secondary lymphoid organs. *Annu Rev Immunol, 26*, 627-650. doi:10.1146/annurev.immunol.26.021607.090257

Rennick, D., Yang, G., Muller-Sieburg, C., Smith, C., Arai, N., Takabe, Y., & Gemmell, L. (1987). Interleukin 4 (B-cell stimulatory factor 1) can enhance or antagonize the factor-dependent growth of hemopoietic progenitor cells. *Proc Natl Acad Sci U S A, 84*(19), 6889-6893.

Riley, J. K., Heeley, J. M., Wyman, A. H., Schlichting, E. L., & Moley, K. H. (2004). TRAIL and KILLER are expressed and induce apoptosis in the murine preimplantation embryo. *Biol Reprod, 71*(3), 871-877. doi:10.1095/biolreprod.103.026963

Roozendaal, R., Mempel, T. R., Pitcher, L. A., Gonzalez, S. F., Verschoor, A., Mebius, R. E., . . . Carroll, M. C. (2009). Conduits mediate transport of low-molecular-weight antigen to lymph node follicles. *Immunity, 30*(2), 264-276. doi:10.1016/j.immuni.2008.12.014

Sabin, F. R. (1916). The Method of Growth of the Lymphatic System. *Science, 44*(1127), 145-158. doi:10.1126/science.44.1127.145

Schafer-Somi, S. (2003). Cytokines during early pregnancy of mammals: a review. *Anim Reprod Sci, 75*(1-2), 73-94.

Schramm, G., Gronow, A., Knobloch, J., Wippersteg, V., Grevelding, C. G., Galle, J., . . . Doenhoff, M. J. (2006). IPSE/alpha-1: a major immunogenic component secreted from Schistosoma mansoni eggs. *Mol Biochem Parasitol, 147*(1), 9-19. doi:10.1016/j.molbiopara.2006.01.003

Schramm, G., Hamilton, J. V., Balog, C. I., Wuhrer, M., Gronow, A., Beckmann, S., . . . Doenhoff, M. J. (2009). Molecular characterisation of kappa-5, a major antigenic glycoprotein from Schistosoma mansoni eggs. *Mol Biochem Parasitol, 166*(1), 4-14. doi:10.1016/j.molbiopara.2009.02.003

Siegert, S., & Luther, S. A. (2012). Positive and negative regulation of T cell responses by fibroblastic reticular cells within paracortical regions of lymph nodes. *Front Immunol, 3*, 285. doi:10.3389/fimmu.2012.00285

Stein, M., Keshav, S., Harris, N., & Gordon, S. (1992). Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med, 176*(1), 287-292.

Sun, R., Urban, J. F., Jr., Notari, L., Vanuytsel, T., Madden, K. B., Bohl, J. A., . . . Shea-Donohue, T. (2016). Interleukin-13 Receptor alpha1-Dependent Responses in the Intestine Are Critical to Parasite Clearance. *Infect Immun, 84*(4), 1032-1044. doi:10.1128/IAI.00990-15

Svensson, L., Arvola, M., Sallstrom, M. A., Holmdahl, R., & Mattsson, R. (2001). The Th2 cytokines IL-4 and IL-10 are not crucial for the completion of allogeneic pregnancy in mice. *J Reprod Immunol, 51*(1), 3-7.

Thieu, V. T., Nguyen, E. T., McCarthy, B. P., Bruns, H. A., Kapur, R., Chang, C. H., & Kaplan, M. H. (2007). IL-4-stimulated NF-kappaB activity is required for Stat6 DNA binding. *J Leukoc Biol, 82*(2), 370-379. doi:10.1189/jlb.1106707

Tontonoz, P., Nagy, L., Alvarez, J. G., Thomazy, V. A., & Evans, R. M. (1998). PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell, 93*(2), 241-252.

Ul-Haq, Z., Naz, S., & Mesaik, M. A. (2016). Interleukin-4 receptor signaling and its binding mechanism: A therapeutic insight from inhibitors tool box. *Cytokine Growth Factor Rev, 32*, 3-15. doi:10.1016/j.cytogfr.2016.04.002

van de Pavert, S. A., & Mebius, R. E. (2014). Development of secondary lymphoid organs in relation to lymphatic vasculature. *Adv Anat Embryol Cell Biol, 214*, 81-91. doi:10.1007/978-3-7091-1646-3_7

Vazquez, M. I., Catalan-Dibene, J., & Zlotnik, A. (2015). B cells responses and cytokine production are regulated by their immune microenvironment. *Cytokine, 74*(2), 318-326. doi:10.1016/j.cyto.2015.02.007

Vicetti Miguel, R. D., Quispe Calla, N. E., Dixon, D., Foster, R. A., Gambotto, A., Pavelko, S. D., . . . Cherpes, T. L. (2017). IL-4-secreting eosinophils promote endometrial stromal cell proliferation and prevent Chlamydia-induced upper genital tract damage. *Proc Natl Acad Sci U S A, 114*(33), E6892-E6901. doi:10.1073/pnas.1621253114

Voehringer, D., Shinkai, K., & Locksley, R. M. (2004). Type 2 immunity reflects orchestrated recruitment of cells committed to IL-4 production. *Immunity, 20*(3), 267-277.

Wegmann, T. G., Lin, H., Guilbert, L., & Mosmann, T. R. (1993). Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today, 14*(7), 353-356. doi:10.1016/0167-5699(93)90235-D

Wery-Zennaro, S., Letourneur, M., David, M., Bertoglio, J., & Pierre, J. (1999). Binding of IL-4 to the IL-13Ralpha(1)/IL-4Ralpha receptor complex leads to STAT3 phosphorylation but not to its nuclear translocation. *FEBS Lett, 464*(1-2), 91-96.

Westerga, J., & Timens, W. (1989). Immunohistological analysis of human fetal lymph nodes. *Scand J Immunol, 29*(1), 103-112.

White, M. F. (1996). The IRS-signalling system in insulin and cytokine action. *Philos Trans R Soc Lond B Biol Sci, 351*(1336), 181-189. doi:10.1098/rstb.1996.0015

Yanagida, M., Fukamachi, H., Ohgami, K., Kuwaki, T., Ishii, H., Uzumaki, H., . . . Nakahata, T. (1995). Effects of T-helper 2-type cytokines, interleukin-3 (IL-3), IL-4, IL-5, and IL-6 on the survival of cultured human mast cells. *Blood, 86*(10), 3705-3714.

Yoshimoto, T. (2018). The Hunt for the Source of Primary Interleukin-4: How We Discovered That Natural Killer T Cells and Basophils Determine T Helper Type 2 Cell Differentiation In Vivo. *Front Immunol, 9*, 716. doi:10.3389/fimmu.2018.00716

Yoshimoto, T., Min, B., Sugimoto, T., Hayashi, N., Ishikawa, Y., Sasaki, Y., . . . Nakanishi, K. (2003). Nonredundant roles for CD1d-restricted natural killer T cells and conventional CD4+ T cells in the induction of immunoglobulin E antibodies in response to interleukin 18 treatment of mice. *J Exp Med, 197*(8), 997-1005. doi:10.1084/jem.20021701

Yoshimoto, T., Yasuda, K., Tanaka, H., Nakahira, M., Imai, Y., Fujimori, Y., & Nakanishi, K. (2009). Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat Immunol, 10*(7), 706-712. doi:10.1038/ni.1737

Zhao, S., Wehner, R., Bornhauser, M., Wassmuth, R., Bachmann, M., & Schmitz, M. (2010). Immunomodulatory properties of mesenchymal stromal cells and their therapeutic consequences for immune-mediated disorders. *Stem Cells Dev, 19*(5), 607-614. doi:10.1089/scd.2009.0345

CHAPTER 2. IL-4 PROMOTES STROMAL CELL EXPANSION AND IS CRITICAL FOR DEVELOPMENT OF A TYPE-2, BUT NOT A TYPE 1 IMMUNE RESPONSE

Modified from: Cortes-Selva, D., Ready, A., Gibbs, L., Rajwa, B., & Fairfax, K. C. (2018). IL-4 promotes stromal cell expansion and is critical for development of a type-2, but not a type 1 immune response. *Eur J Immunol*. doi:10.1002/eji.201847789

Introduction

Secondary lymphoid organs (SLOs) are essential for the development of immune responses; they are responsible for facilitating pathogen and lymphocyte interactions in order to mount an efficient anti-pathogen response, and for supplying different immune-modulatory factors (Junt, Scandella, & Ludewig, 2008; Ruddle & Akirav, 2009). The functions mediated by SLOs rely heavily on the structural organization of the different microenvironments. The complex micro-architecture of SLOs is characterized by the cortex (where B cell follicles are located) and paracortex (containing the T cell zone). In addition, an elaborate network of stromal cells provides both structural support and regulatory signals that trigger B and T cell migration to their respective compartments. Among these mesenchymal (stromal) cells, follicular dendritic cells (FDCs) support B cell survival and proliferation. Previous studies have shown that FDCs produce CXCL13 to attract B cells to the B cell follicle (Klaus, Humphrey, Kunkl, & Dongworth, 1980); where they also trap immune complexes via the complement receptors CR1 (CD35) and CR2 (CD21) (Reynes et al., 1985), thus rendering FDCs essential for germinal center (GC) formation and function. Fibroblastic reticular cells (FRC) have been reported to play an equivalent role in T cell maintenance and B cell survival (Cremasco et al., 2014; Katakai & Kinashi, 2016). FRCs express podoplanin (gp38), produce the chemokines CCL19 and CCL21, which promote the migration of CCR7 expressing cells, as well as IL-7 to promote naïve T cell survival (Farr et al., 1992; Luther,

Tang, Hyman, Farr, & Cyster, 2000; Onder et al., 2012), and Baff to promote B cell survival (Cremasco et al., 2014). Additionally, lymphatic endothelial cells (LEC) secrete CXCL12 and CCL21 and induce recruitment of dendritic cells to the lymph node (Kedl et al., 2017), and their activation by Lymphoid Tissue inducer (LTi) cells has been shown to be critical for the initiation of lymph node development (Onder et al., 2017). Furthermore, it is well established that in lymph nodes the lymphotoxin (LT) $\alpha 1\beta 2$-LTβR pathway is important for both lymph node genesis and proper cellular organization during steady state (McCarthy et al., 2006; Ngo et al., 1999; Onder et al., 2013). Moreover, LT $\alpha 1\beta 2$ mediates the cross-talk between lymphocytes and mesenchymal-derived cells, which is required to maintain lymph node homeostasis and antigen induced expansion (Kain & Owens, 2013; Kumar et al., 2015)

IL-4 is regarded as a pleiotropic cytokine and regulates maturation and survival of B cells, differentiation of Th2 lymphocytes, polarization of macrophages to the alternatively activated (M2) phenotype, and immunoglobulin isotype switching to IgG1/IgE in mice and IgG4/IgE in humans (Coffman et al., 1986; Mosmann, Bond, Coffman, Ohara, & Paul, 1986; Vitetta et al., 1985). IL-4 is broadly produced by T cells, mast cells, basophils, eosinophils, and NKT cells (Brown & Hural, 1997; Pelly et al., 2016; Stetson et al., 2003). IL-4 exerts its function by binding to type I and type II receptor. The type I receptor consist of a widely expressed IL-4Rα and a more constricted common γ-chain. IL-4Rα is expressed on T cells, B cells, eosinophils, macrophages, endothelial cells, fibroblasts and myeloid derived suppressor cells (Heng, Painter, & Immunological Genome Project, 2008; Nelms, Keegan, Zamorano, Ryan, & Paul, 1999). The binding of IL-4 to IL-4R initiates a signaling cascade characterized by phosphorylation of activator of transcription factor-6 (STAT6) and transcription of IL-4 responsive genes (Kammer et al., 1996). Although IL-4, and IL-13 are considered closely related type 2 cytokines, recent reports

have shown that only IL-4 is expressed in the lymph node (Liang et al., 2011; Takeda et al., 1996), which suggests a unique role of IL-4 in these organs. While previous reports on IL-4 have predominantly focused on its role in a Th2 immune response, the potential role of IL-4 in the spatial organization of peripheral lymph nodes microenvironments during homeostasis and antigenic challenge has not been explored.

Given the importance of the IL-4 signaling pathway in T and B cells, and the relevance of lymph node organization in both innate and adaptive immune responses, this study sought to explore the role of the IL-4/IL-4Rα pathway in the maintenance of lymph node (LN) architecture during both homeostasis and following antigenic challenge. During steady state a lack of IL-4 signaling resulted in a 50-70% reduction in FDCs and LECs and disorganization of B cell zones. We then asked whether IL-4 was responsible for LN reorganization in response to prototypical Type 1 (STag) and Type 2 (SEA) antigens. Interestingly, IL-4 was required for SEA induced LN re-organization, lymphotoxin production and germinal center formation, while STag induction of these responses was IL-4 independent. Additionally, The IL-4 independent STag response was shown to be mediated at least partially by IFNγ. Finally, we asked if IL-4 is required for the development of humoral immunity in response to a Tetanus/Diphtheria commercial vaccine. Strikingly immunization of IL-4Rα deficient mice with tetanus/diphtheria failed to induce anti-diphtheria IgG1 while anti tetanus responses were unaffected. Together, our results establish a previously unexplored role of IL-4 in the maintenance of peripheral lymphoid organ microenvironments and the induction of adaptive immunity.

Results

Maintenance of stromal cell networks in peripheral lymph nodes depends on IL-4 signaling pathway

While IL-4 is a cytokine with pleiotropic functions, (Luzina et al., 2012; Paul, 1991) a functional role for IL-4 in the maintenance of the microarchitecture in lymphoid organs in homeostasis has not been explored. Our previous work demonstrated that IL-4 is produced by T follicular helper cells under both Th1 and Th2 inducing conditions (Fairfax et al., 2015). To explore a potential role of IL-4 in peripheral lymph node organization we first examined lymphatic endothelial cells (LEC: $CD31^+$ $PDPN^+$(Thomas et al., 2012)), since they promote lymph node organogenesis, from IL-4 deficient mice by flow cytometry. For this purpose, we used the 4get homozygous (IL-4 GFP reporter mice bred on a BALC/c background (2)), 4get IL-4Rα deficient mice, and mice deficient in IL-4 or STAT6 on a C57BL/6J background. We observed significantly reduced frequency of LEC in mice deficient in multiple components of the IL-4 signaling pathway on both the BALB/c and C57BL/6J backgrounds ($IL\text{-}4^{-/-}$, 4getIL-4R$\alpha^{-/-}$ and $STAT6^{-/-}$), with a 50% reduction in 4getIL-$4R^{-/-}$ mice and 78% and 50% reduction in $IL\text{-}4^{-/-}$ and $STAT6^{-/-}$ respectively, compared to wild-type (Figure 2.1) and an effect size (calculated by Cohen's D) of 1.99, 1.39, and 3.96 in $IL4^{-/-}$, $STAT6^{-/-}$, and 4getIL-4R$\alpha^{-/-}$ respectively. Blood endothelial cell (BEC: CD31+PDPN-) and pericyte (double negative) populations were not significantly altered, while fibroblastic reticular cells (FRC: PDPN+CD31-) trended towards reduction, but the alterations were not statistically significant in any of the 3 strains deficient in IL-4 signaling ($p=0.07$). Moreover, the observed reduction of LEC was limited to popliteal lymph nodes, as we did not observe any significant reduction of LEC in mesenteric lymph nodes (Data not shown). The ImmGen database indicates that LECs have *il4ralpha* transcripts, and we confirmed that they are

also surface positive for IL-4Rα compared to 4getIL-4Rα$^{-/-}$ mice (Figure 2.4). We then quantified the gene expression levels of *CCL19* and *CCL21*, which are both secreted by FRC to induce lymph node entry of lymphocytes, and observed no significant change in fold expression (Figure 2.4). Next, we evaluated the localization of these stromal cells in the popliteal lymph node using tile confocal microscopy (Figure 2.2). Lymphatic endothelial cells are characterized in the lymph node by expression of markers such as pdpn (gp38), CD31, and their location. LEC localize at the subcapsular sinus and come in contact with subcapsular macrophages (Card, Yu, & Swartz, 2014; Yeo & Angeli, 2017). LEC then ramify to the cortex and into the T cell zone and connect to the medulla at the exit of the lymph node. In homeostasis, these cell networks have a distinct structure characterized by abundant invaginations that are interconnected by several junctions; we analyzed the distribution of CD31^{+} and PDPN^{+} endothelial cells and observed a marked mis-localization in 4getIL-4Rα$^{-/-}$ compared to wild-type controls. In the lymph node of wild-type mice, CD31^{+} cells were distributed along the subcapsular sinus, the border of the cortex, and throughout the T cell zone, consistent with previous reports of their steady-state distribution (Mueller & Germain, 2009; Tamburini, Burchill, & Kedl, 2014) (Figure 2.2). In contrast, our mice deficient in IL-4Rα exhibited a collapsed phenotype, with overall less CD31 staining and concentrated clusters of CD31^{+} cells constrained within the medulla, suggesting a requirement of IL-4 for the localization of cortical LECs in the inactivated peripheral lymph node. In order to quantify the spatial distribution of co-stained CD31 and podoplanin, we used analysis of locally bright features (LBF (Knowles, Sudar, Bator-Kelly, Bissell, & Lelievre, 2006)) (Figure 2.3) and observed that LBF in WT were localized near the boundaries the Region of Interest (ROI), whereas the IL-4RαKO trended toward a higher proportion of LBF localized further from the ROI suggesting an altered distribution in the lymph node. We also stained with ICAM-2, a key cellular adhesion molecule

involved in lymphocyte recruitment into the lymph node (van Buul, Kanters, & Hordijk, 2007), in wild-type mice ICAM-2 co-localized with many $CD31^+$ structures in the subcapsular and cortex regions. In contrast, 4getIL-4R$\alpha^{-/-}$ mice have ICAM-2^+ cells in the collapsed $CD31^+$ structures in the medulla, with many cells also staining positive for PDPN (Figure 2.2). Altered distribution of LEC populations did not lead to $CD4^+$ T cell frequencies, but we did observe reduction in T cell number in the IL-4 deficient animals, but not in the STAT6$^{-/-}$ or the 4getIL-4R$\alpha^{-/-}$ mice (Figure 2.5). High endothelial venules (HEV) are responsible for lymphocyte migration to the lymph node (Moussion & Girard, 2011). To confirm that disorganization is not driven by a defect in HEV mediated migration, we analyzed $PNAd^+$ HEV and found no alterations to PNAd localization in 4getIL-4R$\alpha^{-/-}$ mice, indicating that the lack of IL-4 signaling does not alter high endothelial venules (Figure 2.5).

IL-4 signaling is critical for proper follicular dendritic cell positioning in the lymph node

Follicular dendritic cells (FDC) express CXCL13 and capture and present opsonized antigens to B cells (Aguzzi, Kranich, & Krautler, 2014; El Shikh & Pitzalis, 2012). MFG-E8 (suggested to be identical to FDC marker, FDC-M1) and FDC-M2 (activated C4, (Allen & Cyster, 2008)) are highly expressed in this cell population and in primary and secondary follicles and are considered specific molecular markers of FDC. In addition, FDC are recognized by antibodies to the complement receptors CD21 and CD35 (37). In our experiments, we observed a significant decrease in the frequency and cell numbers of FDC-$M2^+$ $CD21/35^+$ FDC cells in mice lacking the IL-4 signaling cascade (IL-$4^{-/-}$, 4get/IL-4R$\alpha^{-/-}$ and STAT6$^{-/-}$) on both the C57BL/6J and BALB/c genetic backgrounds (Figure 2.7). As with LECs, the bulk population of FDCs are surface positive for IL-4Rα (Figure 2.7). As FDCs have previously been reported to maintain B cell area via CXCL13 (Aguzzi et al., 2014), we investigated the CXCL13 gene expression in peripheral lymph

nodes of 4getIL-4R$^{-/-}$ mice, and observed no alterations (Figure 2.7). Using tile confocal microscopy, we observed clusters of FDC-M2^{+} and CD21/35^{+} cells on the edges of the B cell zones in 4get homozygous mice, as expected. We also observed B cell follicle mis-localization in IL-4Rα deficient mice, with B cell follicles localized further from capsule in compared to wild type (Figure 2.8). B cell follicle mis-localization was not related to change in cell number, as we observed no significant difference in frequencies or cell number on either the BALB/c or C57BL/6J background (Figure 2.6). The IL-4Rα deficient mice on the other hand, displayed distinctly altered localization of FDCs (characterized as FDC-M2^{+} in Fig 2F and CD21/35^{+} in Fig. 2G), with B cells clustering around the aberrantly positioned FDCs. Moreover, we analyzed the spatial positioning of CD21/35^{+} FDC by quantification of distance to capsule (normalized to the total area of the LN) and observed significant alterations in the positioning of FDC in the IL-4RαKO mice in comparison to wild type controls (Figure 2.8). Interestingly, FDCs and B cells were consistently found clustered together, since bulk CXCL13 expression is unaltered, it suggests that IL-4 mediated positioning is independent of CXCL13. These data suggest that the stromal-lymphocyte organizational axis (Bajenoff et al., 2006; Zhou et al., 2003) is compromised in mice in which the IL-4 signaling pathway is deficient in some of its components, a state which could potentially lead to a deficiency in the B cell response to antigenic challenge.

Deficiency of IL-4 correlates with downregulation of lymphotoxin β and lymphotoxin α gene expression

During homeostasis, the development and regulation of diverse lymphoid tissue microenvironments is tightly related to expression of lymphotoxin. Mice deficient in LTα, LTβ, and LTβR all present defects in lymphoid organogenesis, showing the remarkable dependence of lymphoid homeostasis on the LTα1β2- LTβR signaling axis (Zindl et al., 2009). In addition, BAFF

(known as BlyS, TALL-1, TNFSF13B, TNFSF20) is secreted by FRCs and FDCs and also plays a role in the maintenance of LN architecture by aiding in the viability of B cells (Cremasco et al., 2014). We investigated whether the disorganization observed in peripheral lymphoid organs could be due to defects in the LTα1β2- LTβR signaling pathway, or BAFF. For this, we first tested the gene expression of *ltβ* and *lta* in mice lacking IL-4Rα or the transcription factor Stat6. Remarkably, absence of IL-4Rα correlated to significant decrease in both *ltα* and *ltβ* gene expression. Nevertheless, mRNA expression of *baff* in the lymph node was unaffected in the absence of IL-4R*a* (Figure 2.9). Similarly, we also tested the gene expression of *ltβ* and *ltα* in popliteal lymph nodes extracted from C57BL/6J mice, IL-$4^{-/-}$, and in $STAT6^{-/-}$ mice; we observed significantly reduced gene expression of *ltα* and *ltβ* , but not *baff* in the $STAT6^{-/-}$ and IL-$4^{-/-}$ animals (Figure 2.9). Furthermore, protein expression of LT*β* was also down-regulated in IL-4R deficient and STAT6 knockout mice compared to their respective wild types (Figure 2.9 Panel C). These data suggest that IL-4 may act in an IL-4R and Stat-6 dependent manner to sustain LTα/β production but is not involved in regulating BAFF.

IL-4 is required for LN re-organization and GC formation in a Type-2 but not a Type-1 response

The organization and expansion of B and T cells in lymph nodes following antigenic stimulation is closely related to the complex stromal cell network, (Bajenoff et al., 2006) therefore we wondered if the disorganization observed in stromal cells from peripheral lymph nodes affected the adaptive immune response to the parasitic antigens of *Schistosoma mansoni* (SEA, which induces a Type 2 response), and *Toxoplasma gondii* antigen (STag, which induces a Type 1 response).

To determine whether IL-4 deficiency affected the expansion of T follicular helper cells (defined as $CD4^{+}CXCR5^{+}PD\text{-}1^{+}$) in peripheral lymph nodes we injected the footpad of $IL\text{-}4^{-/-}$ mice and C57BL/6 mice with unadjuvanted SEA and STag. Similar to what we have previously published (Fairfax et al., 2015), immunization with SEA resulted in reduced Tfh in $IL\text{-}4^{-/-}$ (but with no reduction in Tfh cell function as measured by IL-21 production, Figure 2.10) and plasma cells, as compared to wildtype controls at 8-days post-immunization (Figure 2.10 C). While the lack of IL-4 did not lead to diminished Tfh or plasma cells following STag immunization (Figure 2.10 A, C). As expected, $IL\text{-}4^{-/-}$ mice immunized with either SEA or STag had significantly reduced production of IgG1 (Figure 2.11). Given that Tfh cells have been shown to be positional-dependent cells (Chen, Ma, Zhang, Wu, & Qi, 2015), we investigated the role of IL-4 in the organization of these cells in the lymph node. We imaged B and T cell zones as well as germinal centers in fresh-frozen sections of reactive lymph nodes at day 8 post-immunization. Naïve IL-4 deficient mice showed the characteristic disorganization of B cell follicles as observed in Figure 2.8 (Figure 2.12), with B220 positive cells distributed along the paracortex, cortex and medulla, as well as a loss of defined B-cell follicles. Mice immunized with SEA also showed a marked disorganization of the B cell follicles and T cell zone, with B cell clusters appearing throughout the T cell zone and no apparent organized GL7 positive germinal centers (Figure 2.12), whereas mice immunized with STag showed organization and germinal center localization similar to the wild type control. (Figure 2.12 B). The analysis of spatial positioning of $B220^{+}$ cells by quantification of distance to capsule (normalized to the total area of the LN) showed that IL-4 knockout mice at both steady state, and immunized with SEA antigens have B cell follicles localized significantly further from capsule in comparison to wild type, whereas IL-4 knockout mice immunized with STag exhibit similar localization of B cell follicles from the capsule when compared to wild type controls

(Figure 2.12 B). Since we identified defects in lymphotoxin expression at steady state in the absence of IL-4 signaling, we assessed the ability of SEA and STag to induce *ltβ* and *ltα* gene expression in the absence of IL-4. SEA immunization of IL-4$^{-/-}$ mice induced significantly less *ltβ* and *ltα* expression as compared to wildtype mice, while STag immunization induced equal transcripts levels of *ltβ* and *ltα* in wildtype and IL-4$^{-/-}$ animals (Figure 2.12 E), suggesting that there is an IL-4 independent mechanism for lymph node re-organization during a Type 1 immune response.

IFNγ mediates LEC expansion in the absence of IL-4

Our data indicate that STag immunization is able to induce lymphotoxin production and lymph node reorganization in the absence of IL-4. Since we have previously shown that STag induces significantly more IFNγ expression than SEA does (Fairfax et al., 2015), we wondered if IFNγ production is the mechanism through which STag is able to stimulate LEC and FDC expansion and lymph node re-organization independently of IL-4. To examine this possibility, we administered blocking anti-IFNγ mAb or isotype control to 4get and 4getIL-4Rα$^{-/-}$ mice (4getIL-4Rα$^{-/-}$ have significantly reduced FDCs and LEC at steady state) beginning at three days before immunization with STag (Figure 2.13). At day 8 post immunization, isotype control treated STag immunized 4get and 4get IL-4Rα $^{-/-}$ mice have equivalent cell numbers of LECs (Figure 2.13, p=0.1299) and FDCs (Figure 5C, p=0.3841), suggesting that STag immunization induces expansion of these populations in 4get IL-4Rα $^{-/-}$ mice. Anti-IFNγ mAb treatment leads to significantly fewer LECs in 4get IL-4Rα $^{-/-}$ and in control 4get mice (Figure 2.13 B), indicating that STag induced expansion of lymphatic endothelial cells is at least partially IFNγ-dependent in the absence of IL-4Rα signaling. Anti-IFNγ mAb treatment significantly reduced the frequency

and number of FDCs in control 4get, but only reduced FDC frequency in 4get IL-4Rα $^{-/-}$ mice (Figure 2.13 C), suggesting that in the absence of IL-4, FDC expansion is not significantly dependent on IFN-γ and that the two stromal cell populations have different requirements for T-cell derived cytokines.

IL-4 is required for the development of a humoral immune response to tetanus/diphtheria vaccination

Our experiments with SEA as a model Type 2 antigenic challenge suggested that the development of type 2 immunity is compromised in the absence of IL-4 signaling. Since the majority of commercially available vaccines rely on alum as an adjuvant, we sought to determine if this observation has consequences for the development of humoral immunity to tetanus/diphtheria. To address this, we immunized 4get (IL-4 GFP reporter mice) and 4getIL-4Rα$^{-/-}$ mice s.c. with 1/10 the human dose of tetanus/diphtheria. We have previously established that the peak of the Tfh cell response (to both Th1 and Th2 antigens (Fairfax et al., 2015)) occurs at day 8-post immunization, therefore we assessed Tfh cell populations and IL-4 secretion at day 8 post-immunization. At this time-point 4getIL-4Rα$^{-/-}$ mice have a 60% reduction in the frequency of Tfh cells and a 70% reduction in GFP^{+} Th2 cells accompanied by a significant reduction in cell number (Figure 2.14 A and B) compared to wildtype 4get mice. We next explored the B cell response at day 14 (the peak of the germinal B cell reaction under both Th1 and Th2 conditions). At this time-point the early loss of Tfh cells is accompanied by a 60-70% reduction in germinal center and plasma cells in tetanus/diphtheria immunized IL-4Rα deficient mice (Figure 2.14 C and D). We then examined the stromal cell compartment to see if tetanus/diphtheria is able to expand the FDC and LEC populations. Immunized 4getIL-4Rα$^{-/-}$ have significant reduction in the frequency and cell numbers of both LECs and FDCs ($p= 0.0093$ and $p= 0.0217$, Figure 2.15)

compared to immunized 4get control mice. Interestingly, in absence of IL-4, the antibody response to diphtheria is significantly compromised at both days 8 and 14 post immunization, whereas the response to tetanus is not (Figure 2.15 A and B), suggesting that diphtheria has a requirement for IL-4 that is similar to that of SEA. In order to rule out the possibility that impaired Tfh response and humoral responses resulted from intrinsic defects of IL-4R in these cell populations, we analyzed the mesenteric lymph node following tetanus/diphtheria immunization and observed no differences in $CD31^{+}PDPN^{+}$ LEC in IL-4Rα deficient mice in comparison to wild type control. Figure 2.16).

Together, these data suggest that alum induced cellular and humoral immunity to certain antigens is compromised in the absence of IL-4 signaling, a finding that correlates to impaired expansion of certain stromal cell population in the lymph node and has significant translational implications.

Discussion

IL-4 is known to have critical effector and homeostatic effects on lymphocytes, including protecting B cells from apoptosis, inducing IgG1 and IgE production, polarization of $CD4^{+}$ T cells to a Th2 phenotype. For non-lymphocytes, it has been recently suggested that IL-4 acts directly on human mesenchymal progenitors and downregulates gp38 on stromal cells lines, acting as a B-cell extrinsic mechanism to modulate the Tfh/Tfh-like- stroma crosstalk in follicular lymphoma (Pandey et al., 2017). Given the relevance of lymphocyte and stromal cell population in lymphoid organs, we sought to determine the function of IL-4 in peripheral lymph nodes. In the lymph node, IL-4 is recognized primarily as a type 2 cytokine that regulates antibody class-switching. Here we provide evidence that IL-4 production also functions to maintain peripheral lymph node architecture in steady state, and drives antigen induced lymph node expansion. Specifically, we

found that mice deficient in the IL-4 signaling cascade have an altered distribution of B cell follicles, with reduced FDCs and LECs both in steady state and following type 2 antigenic challenge. Our data, from multiple strains of mice deficient in components of the IL-4 signaling pathway, demonstrate that IL-4 supports lymph node lymphotoxin production in steady state, a key lymphokine known to be critical for maintaining the lymphocyte-stromal cell axis. To our knowledge, this is the first report of a role for IL-4 in lymph node homeostasis.

C57BL/6J mice are recognized to have an immune skewing towards IFNγ production and strong type-1 immune responses while BALB/c mice produce more IL-4 and are skewed towards strong type 2 responses, characteristics that affect susceptibility/resistance to multiple pathogens (Mills, Kincaid, Alt, Heilman, & Hill, 2000; Sellers, Clifford, Treuting, & Brayton, 2012). This skewing raised the possibility that IL-4 could have strain specific homeostatic effects. To ensure the broad applicability of our findings we used mice on both BALB/c and C57BL/6J backgrounds. IL-4, IL-4Rα, and Stat-6 deficient mice from both genetic backgrounds all have profound alterations in both the frequency and cell number of LECs, and the positioning of $PDPN^{+}$ and $CD31^{+}$ structures within the peripheral lymph node (Figure 1B, 1C, 1G). LECs have previously been reported to be dependent on lymphotoxin β receptor during inflammatory lymphangiogenesis (Furtado et al., 2007), so, our findings that they express IL-4Rα on their surface, and that IL-$4^{-/-}$, 4getIL-4R$\alpha^{-/-}$, and Stat$6^{-/-}$ mice all have reduced transcripts of both LTα and LTβ, suggests that the effects of IL-4 deficiency may be due in part to altered production of these key cytokines. Helminth infection has been previously shown to induce fibroblastic reticular cell driven *de novo* B cell follicle formation in the mesenteric lymph node (MLN) via an IL-4Rα dependent induction of lymphotoxin (Dubey et al., 2016). Those experiments did not find a defect in MLN lymph node

organization in IL-4Rα$^{-/-}$ at steady state, but the authors did not examine peripheral lymph nodes. We have examined the mesenteric lymph nodes of both IL-4$^{-/-}$ and 4getIL-4Rα$^{-/-}$ mice and have not observed the same disorganization that we have found in peripheral lymph nodes (Supplemental Figure 1 and data not shown), suggesting that homeostatic cytokine signals are distinct in different lymphoid organs, as has been previously suggested by other groups. Previous work has demonstrated that blockade of LTαβ/LTβR interactions resulted in spleen and Peyer's patch development, but not lymph development (Ettinger, Browning, Michie, van Ewijk, & McDevitt, 1996). Additional work by Koni et al. showed that complete absence of LTβ in mice translates in defective organization of peripheral lymph nodes, but not of mesenteric lymph nodes (Koni et al., 1997), while Banks et al. observed that LTα deficiency correlated with defective peripheral lymph nodes and Peyer's patches, but that some lymph node-like structures were found in the mesenteric fat (Banks et al., 1995). In addition, Ettinger et al. showed a dose- dependent effect of blocking of LTβR in splenic development (Ettinger et al., 1996). All of these data suggest differential signal requirements across secondary lymphoid organs, and that varying levels of cytokines results in fluctuating effects in lymphoid organization. It is possible that in our model the downregulation of expression of LTβ and LTα is not enough to cause a defect in mesenteric or that other compensatory signal(s) unique to the mesenteric lymph are required. Furthermore, mesenteric lymph nodes are known to have constitutive germinal centers due in part to signals from the microbiota (Round & Mazmanian, 2009). Given our data that indicate that STag induced IL-4 independent lymph node re-organization is at least partially dependent on IFNγ (Figure 5A-C), it is plausible that commensal microbiota induction of cytokines is a mechanism behind the difference between the MLN and peripheral lymph nodes in steady state. In our model IFNγ blockade with a neutralizing antibody was able to reduce STag induced peripheral lymph node

LEC expansion, but not FDC expansion, which suggests that while the two cell types have an equivalent requirement for IL-4 signaling, they differ in their dependence on IFNγ for antigen induced expansion. This raises the possibility that different stromal cell subsets may be regulated by different combinations of B and T-cell derived cytokines, a possibility that will be explored in future work. We also observed profoundly altered localization of B cell follicles in IL-$4^{-/-}$ and 4getIL-$4R^{-/-}$ mice, an observation that is in agreement with previous reports that *K14-VEGFR-3-Ig* mice that lack lymphatic capillaries have disorganized B cell zones in their peripheral lymph nodes (Thomas et al., 2012), and supports the notion that stromal cells are critically important for maintaining peripheral lymph node homeostasis and lymphocyte positioning.

Our experiments also revealed a significant decrease in the number of FDC-$M2^{+}$ follicular dendritic cells in the absence of IL-4 signaling (Figure 2B, C), and even more strikingly, a profound aberration in their localization within peripheral lymph nodes (Figure 2F, G). In our model, the decreased number of follicular dendritic cells did not correlate with reduced B cell numbers in steady state or *Baff* transcript levels, suggesting that IL-4 influenced FDC activation or function does not compromise B cell development. It is unclear whether other B cell survival signals such as Bcl-2 or transcription factors of the NF-kB family, which have been shown as crucial for B cell (Gerondakis & Strasser, 2003) play a compensatory role in the maintenance of B cells. Interestingly, the aberrantly located FDCs co-localized with clusters of $B220^{+}$ B cells, raising the possibility that this FDC mal-positioning drives alterations to B cell follicles, a possibility that is supported by published data that indicate FDCs and B cells maintain their positioning via a LTαβ-LTβR positive feedback loop (Ansel et al., 2000; Boulianne, Porfilio, Pikor, & Gommerman, 2012; Mackay, Majeau, Lawton, Hochman, & Browning, 1997). Nevertheless, the source of LTαβ in these studies remain unknown, and will be the focus of future

studies. CXCL13 production is not altered in the absence of IL-4, suggesting that the traditional mechanisms underlying B cell positioning are intact, and supports the possibility that the observed alterations in positioning are likely driven by inappropriate positioning of stromal cells. The relative contributions of FDCs versus LECs in governing B cell positioning remains unclear, however, the data suggest that both of these stromal cell populations are responsive to both IL-4 (Figures 1 and 2) and lymphotoxin (Chaplin & Fu, 1998; Dubey et al., 2016), and that alterations to either cell type alters B cell follicle localization. This raises the possibility that LECs and FDCs work in concert to enforce peripheral lymph node organization.

Overall our data suggest a novel role for IL-4 in maintaining the lymphocyte stromal axis in peripheral lymph nodes both during steady state, and following antigen induced inflammation. This is due in part to IL-4 induced production of lymphotoxin-α and β, but it is likely that there are other molecular mechanisms involved in this regulation including a cell intrinsic role for IL-4 signaling in FDCs and LECs, and the mechanistic delineation of the stromal cell intrinsic versus extrinsic roles of IL-4 will be the focus of future work. Our work used Stat-6 and IL-4Rα deficient mice to complement the findings from IL-4 deficient mice. Both of these strains would also be deficient in responding to IL-13. We looked for IL-13 expression at steady state, but were unable to detect significant transcripts of *il-13* in naïve peripheral lymph nodes on any genetic background (data not shown), While this is not conclusive, it suggests that physiological relevance is unlikely in our model. Future studies should strive to unequivocally determine the homeostatic role of IL-13 in the maintenance of stromal cell population in peripheral lymph nodes.

Our experiments found that a commercially available tetanus diphtheria vaccine failed to induce significant expansion of either FDC of LEC in IL-4Rα deficient mice. Furthermore, when we examined the cellular and humoral immune response to tetanus/diphtheria, we found that in

the absence of IL-4 signaling, immunization fails to induce robust Tfh and germinal center formation and most significantly, fails to induce plasma cell differentiation. This is accompanied by significantly lower serum anti-diphtheria IgG1 titers, a finding that is likely to have clinical consequences. FDCs have previously been shown to important for the maintenance of memory B cells and long-term humoral immunity (Ahearn et al., 1996; Fang, Xu, Fu, Holers, & Molina, 1998) (via CD21/35), so these data fit within that paradigm. The inability of IL-4Rα deficient mice to undergo lymph node re-organization following immunization with some Th2 antigens is particularly important because most commercially available vaccines are administered s.c. or i.m. and would drain to peripheral lymph nodes. The majority of these formulations utilize aluminum salts as their adjuvants, and these compounds are known induce a strong Th2 response (Brewer, 2006; Lindblad, 2004). The reduction in diphtheria, but not tetanus specific responses suggests that individual antigens may have an intrinsic requirement for IL-4 induction regardless of the adjuvant that they are administered with. This possibility has significant implications for monitoring the efficacy of vaccine formulations with multiple antigens, and for understanding the molecular basis of immunization failure. A greater understanding of the role of cytokines like IL-4 and IFNγ in lymph node organization/reorganization may lead to rational approaches to promote long term humoral immunity.

Materials and methods

Mice strains and *in vivo* treatments

C57BL/6J (WT), C57BL/6J IL-4$^{-/-}$, C57BL/6J STAT6$^{-/-}$ 4get homozygous (Jackson Laboratory), and 4get IL-4Rα$^{-/-}$ (gift from Markus Mohrs) were bred in-house under specific pathogen free (SPF) conditions at Purdue University. All experimental procedures with mice were approved by the Purdue Animal Care and Use Committee (PACUC). All experimental mice were

a mix of female and male age-matched mice of approximately 6-8 weeks of age. For immunization experiments, SEA was prepared from isolated *Schistosoma mansoni* eggs as previously described (MacDonald et al., 2002; Taylor, Krawczyk, Mohrs, & Pearce, 2009). STag was prepared from culture-derived *Toxoplasma gondii* tachyzoites ((Fairfax et al., 2015) strain ME-49, kindly provided by William Sullivan). Thirty μg of either SEA or STag were injected s.c. in the rear footpad and mice were sacrificed 8-days post-immunization. Tetanus/Diphtheria commercial vaccine (Tetanus and Diphtheria Toxoids Adsorbed, MassBiologics) was injected s.c. in the rear footpad at ~1/10 the human dose. For IFN-γ blockade experiments, 200μg of anti-IFN-γ (XMG1.2, BioXcell (Choi et al., 2015) or isotype control (anti horseradish peroxidase, Clone: HRPN, BioXcell) was injected both s.c. and i.p. on days -3, 0, 1, 3 and 5.

Enzymatic digestion of popliteal lymph nodes

For flow cytometric analysis, collected pLN were digested with DMEM containing 1mg/ml of Collagenase D (Sigma) and 0.1 mg/mL DNAse I (Invitrogen) as previously described (Hara et al., 2012; Ng, Nayak, Schmedt, & Oldstone, 2012). In short, digestion medium was injected in the center of the nodes and incubated at 37°C for 15 min, with occasional inversion to ensure the mixing of the contents. The digested nodes were filtered through a 100 μm cell strainer and cell suspensions were washed with 15 mL of DMEM. Following wash with DMEM, cells were centrifuged (1,500 rpm, 5 min, 4°C), cells were re-suspended to appropriate volume in FACS buffer (2% fetal calf serum (FCS), 5 mM EDTA in PBS), counted for cell numbers and used for flow cytometry staining.

Flow cytometry and antibodies

Surface staining and intracellular staining for single cell suspensions was performed as previously described (Glatman Zaretsky et al., 2009). Samples were acquired using a BD FACSCanto II and analyzed using FlowJo software (TreeStar, v.10.0.6). In brief, intracellular staining was performed by re-stimulation of cells for 5 h with PMA 50 ng/mL) and ionomycin (1mg/mL) in the presence of brefeldin A (10 mg/mL) as described previously (Everts et al., 2012). The following antibodies from BD, eBioscience, Bio-Legend, ImmunoKontact) conjugated with PE, PE-Cy5, PE-Cy7, allophycocyanin, allophycocyanin-Cy7, Pacific blue, or biotin) were used: CD4 (RM4-5), CD19 (1D3), CD138, (281-2), IgG1 (A85-1), IgD (11-26), CD11c (N418), F4/80 (BM8), GL7 (GL7), CD31 (390), podoplanin (eBIO 8.1.1), FDC-M2 (FDC-M2), CD21/35 (7G6), PD-1 (J43), CXCR5 (2G8), PNAd (MECA-79). IL-21R/FC chimera (R&D Systems; 596-MR-100) was used for detection of IL-21–producing cells. Secondary staining was performed using streptavidin- allophycocyanin or streptavidin allophycocyanin-Cy7. To reduce non-specific binding, Fc-block (anti-mouse CD16/32 (clone 93)) was used in all flow cytometry experiments. Flow cytometry experiments were conducted in accordance to the "Guidelines for the use of flow cytometry and cell sorting in immunological studies"(Cossarizza et al., 2017).

Immunofluorescence microscopy

The entire collected lymph node was placed in Tissue-Tek optimum cutting temperature compound (OCT) (Thermo Scientific) and immediately frozen in liquid nitrogen. Serial cryostat sections (10μm) were collected, air-dried and fixed in ice-cold 75% acetone/25% ethanol for 5 mins. Tissue was rehydrated in PBS for 10 min and blocked using biotin blocking kit (Vector Laboratories, according to manufacturer's instructions) followed by incubation with 1%(v/v) in PBS of rat and rabbit serum. Immunofluorescence staining was performed overnight at 4°C with

diluted antibodies in blocking buffer. Secondary staining was performed the following morning by incubating with specific antibodies for 1 hour at room temperature. Mounting was done using ProLong anti-fade reagents (Life Technologies) followed by imaging using Leica TCS Sp5 Laser Scanning Microscopy using tile scan feature with an average grid size of 3x3 taken with a 20x objective at a resolution of 1024x1024. Image post-processing was done using Fiji is Just ImageJ software (1.47v).

RNA isolation and q-RT-PCR analysis

Whole collected lymph nodes were homogenized in Trizol™ and RNA isolation was performed as previously described (Fairfax, Everts, Smith, & Pearce, 2013; Heng et al., 2008). RNA was used for cDNA synthesis using Superscript II (Invitrogen) for qPCR analysis. qPCR for *Ltβ* (Assay ID: Mm00434774_g1), *Ltα* (Assay ID: Mm00440228-gH), *Baff* (Assay ID: Mm00446347_m1), *ccl19* (Assay ID: Mm00839967_g1), *ccl21b* (Assay ID: Mm03646971_gH) and *cxcl13* (Assay ID: Mm00444533_m1) was performed using Taqman Gene expression assays with beta actin as the housekeeping gene (Assay ID: Mm02619580_g1). (ThermoFisher) on an Applied Biosystems StepOne Plus Real-Time PCR System.

Western blot

Total protein was isolated from naïve lymph nodes using Tissue extraction Reagent I (Invitrogen) and diluted in sample buffer. 30 μg of protein was loaded to 15% SDS-PAGE gels and transferred to nitrocellulose membrane. Membranes were incubated with anti-lymphotoxin β antibody (Abcam, ab64835) and β-tubulin antibody (Santa Cruz, clone N-20) overnight and imaged using Odyssey infrared imaging system from LI-COR Biosciences.

ELISA

Tetanus- and diphtheria-specific IgG1 endpoint titers were determined by enzyme-linked immunosorbent assay (ELISA) using the mAb X56 (BD) and Immulon 4HB plates (Thermo Fisher Scientific). Plates were coated overnight with tetanus (List Labs) and diphtheria with 2μg/mL/well at 4°C, blocked with 1% milk and incubated with serial dilutions of sera, followed by incubation with anti-mouse IgG1 ads-HRP antibody and ABTS substrate. Plates were read at 405 nm at room temperature on a BioTek plate reader.

Quantification of Podoplanin/CD31 co-stained areas

To visualize and quantify the spatial distribution of areas co-stained with an antibody against podoplanin and a label identifying CD31, we used Euclidean distance map (EDM) analysis of locally bright features (LBF) following the methodology by Knowles et al. (Knowles et al., 2006). Briefly, the lymph node images were processed to produce a Boolean-and result of podoplanin and CD31 channel. The resultant greyscale images represent co-stained pixels. The images were further automatically threshold to LBFs. Separately, a region of interest (ROI) mapping the boundaries of the lymph nodes were established and converted into binary masks. The mask images were transformed to 16-bit greyscale EDMs. Finally, the EDM-LBF maps were created by multiplying the EDM images by the LBF maps. The results encoded the distances to the boundaries of ROIs as greyscale values. The quantitation was blinded and performed by two different researchers.

Statistical analysis

Statistical analyses were performed using either a non-parametric Mann-Whitney test, unpaired Student's t test or ANOVA, Bonferroni's multiple comparison test based on the

distribution of the data. p values ≤ 0.05 were considered statistically significant. Graph generation and statistical analyses were performed using Prism (GraphPad v5.0). Cohen's d was calculated for effect size based on between-group differences by using the means and standard deviation of two groups. Values >0.8 were considered a large effect (Cohen, 1992).

References

Aguzzi, A., Kranich, J., & Krautler, N. J. (2014). Follicular dendritic cells: origin, phenotype, and function in health and disease. *Trends Immunol, 35*(3), 105-113. doi:10.1016/j.it.2013.11.001

Ahearn, J. M., Fischer, M. B., Croix, D., Goerg, S., Ma, M., Xia, J., . . . Carroll, M. C. (1996). Disruption of the Cr2 locus results in a reduction in B-1a cells and in an impaired B cell response to T-dependent antigen. *Immunity, 4*(3), 251-262.

Allen, C. D., & Cyster, J. G. (2008). Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Semin Immunol, 20*(1), 14-25. doi:10.1016/j.smim.2007.12.001

Ansel, K. M., Ngo, V. N., Hyman, P. L., Luther, S. A., Forster, R., Sedgwick, J. D., . . . Cyster, J. G. (2000). A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature, 406*(6793), 309-314. doi:10.1038/35018581

Bajenoff, M., Egen, J. G., Koo, L. Y., Laugier, J. P., Brau, F., Glaichenhaus, N., & Germain, R. N. (2006). Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity, 25*(6), 989-1001. doi:10.1016/j.immuni.2006.10.011

Banks, T. A., Rouse, B. T., Kerley, M. K., Blair, P. J., Godfrey, V. L., Kuklin, N. A., . . . Mucenski, M. L. (1995). Lymphotoxin-alpha-deficient mice. Effects on secondary lymphoid organ development and humoral immune responsiveness. *J Immunol, 155*(4), 1685-1693.

Boulianne, B., Porfilio, E. A., Pikor, N., & Gommerman, J. L. (2012). Lymphotoxin-sensitive microenvironments in homeostasis and inflammation. *Front Immunol, 3*, 243. doi:10.3389/fimmu.2012.00243

Brewer, J. M. (2006). (How) do aluminium adjuvants work? *Immunol Lett, 102*(1), 10-15. doi:10.1016/j.imlet.2005.08.002

Brown, M. A., & Hural, J. (1997). Functions of IL-4 and control of its expression. *Crit Rev Immunol, 17*(1), 1-32.

Card, C. M., Yu, S. S., & Swartz, M. A. (2014). Emerging roles of lymphatic endothelium in regulating adaptive immunity. *J Clin Invest, 124*(3), 943-952. doi:10.1172/JCI73316

Chaplin, D. D., & Fu, Y. (1998). Cytokine regulation of secondary lymphoid organ development. *Curr Opin Immunol, 10*(3), 289-297.

Chen, X., Ma, W., Zhang, T., Wu, L., & Qi, H. (2015). Phenotypic Tfh development promoted by CXCR5-controlled re-localization and IL-6 from radiation-resistant cells. *Protein Cell, 6*(11), 825-832. doi:10.1007/s13238-015-0210-0

Choi, Y. S., Gullicksrud, J. A., Xing, S., Zeng, Z., Shan, Q., Li, F., . . . Crotty, S. (2015). LEF-1 and TCF-1 orchestrate T(FH) differentiation by regulating differentiation circuits upstream of the transcriptional repressor Bcl6. *Nat Immunol, 16*(9), 980-990. doi:10.1038/ni.3226

Coffman, R. L., Ohara, J., Bond, M. W., Carty, J., Zlotnik, A., & Paul, W. E. (1986). B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J Immunol, 136*(12), 4538-4541.

Cohen, J. (1992). A power primer. *Psychol Bull, 112*(1), 155-159.

Cossarizza, A., Chang, H. D., Radbruch, A., Akdis, M., Andra, I., Annunziato, F., . . . Zimmermann, J. (2017). Guidelines for the use of flow cytometry and cell sorting in immunological studies. *Eur J Immunol, 47*(10), 1584-1797. doi:10.1002/eji.201646632

Cremasco, V., Woodruff, M. C., Onder, L., Cupovic, J., Nieves-Bonilla, J. M., Schildberg, F. A., . . . Turley, S. J. (2014). B cell homeostasis and follicle confines are governed by fibroblastic reticular cells. *Nat Immunol, 15*(10), 973-981. doi:10.1038/ni.2965

Dubey, L. K., Lebon, L., Mosconi, I., Yang, C. Y., Scandella, E., Ludewig, B., . . . Harris, N. L. (2016). Lymphotoxin-Dependent B Cell-FRC Crosstalk Promotes De Novo Follicle Formation and Antibody Production following Intestinal Helminth Infection. *Cell Rep, 15*(7), 1527-1541. doi:10.1016/j.celrep.2016.04.023

El Shikh, M. E., & Pitzalis, C. (2012). Follicular dendritic cells in health and disease. *Front Immunol, 3*, 292. doi:10.3389/fimmu.2012.00292

Ettinger, R., Browning, J. L., Michie, S. A., van Ewijk, W., & McDevitt, H. O. (1996). Disrupted splenic architecture, but normal lymph node development in mice expressing a soluble lymphotoxin-beta receptor-IgG1 fusion protein. *Proc Natl Acad Sci U S A, 93*(23), 13102-13107.

Everts, B., Hussaarts, L., Driessen, N. N., Meevissen, M. H., Schramm, G., van der Ham, A. J., . . . Yazdanbakhsh, M. (2012). Schistosome-derived omega-1 drives Th2 polarization by suppressing protein synthesis following internalization by the mannose receptor. *J Exp Med, 209*(10), 1753-1767, S1751. doi:10.1084/jem.20111381

Fairfax, K. C., Everts, B., Amiel, E., Smith, A. M., Schramm, G., Haas, H., . . . Pearce, E. J. (2015). IL-4-Secreting Secondary T Follicular Helper (Tfh) Cells Arise from Memory T Cells, Not Persisting Tfh Cells, through a B Cell-Dependent Mechanism. *J Immunol, 194*(7), 2999-3010. doi:10.4049/jimmunol.1401225

Fairfax, K. C., Everts, B., Smith, A. M., & Pearce, E. J. (2013). Regulation of the Development of the Hepatic B Cell Compartment during Schistosoma mansoni Infection. *J Immunol*. doi:10.4049/jimmunol.1301357

Fang, Y., Xu, C., Fu, Y. X., Holers, V. M., & Molina, H. (1998). Expression of complement receptors 1 and 2 on follicular dendritic cells is necessary for the generation of a strong antigen-specific IgG response. *J Immunol, 160*(11), 5273-5279.

Farr, A. G., Berry, M. L., Kim, A., Nelson, A. J., Welch, M. P., & Aruffo, A. (1992). Characterization and cloning of a novel glycoprotein expressed by stromal cells in T-dependent areas of peripheral lymphoid tissues. *J Exp Med, 176*(5), 1477-1482.

Furtado, G. C., Marinkovic, T., Martin, A. P., Garin, A., Hoch, B., Hubner, W., . . . Lira, S. A. (2007). Lymphotoxin beta receptor signaling is required for inflammatory lymphangiogenesis in the thyroid. *Proc Natl Acad Sci U S A, 104*(12), 5026-5031. doi:10.1073/pnas.0606697104

Gerondakis, S., & Strasser, A. (2003). The role of Rel/NF-kappaB transcription factors in B lymphocyte survival. *Semin Immunol, 15*(3), 159-166.

Glatman Zaretsky, A., Taylor, J. J., King, I. L., Marshall, F. A., Mohrs, M., & Pearce, E. J. (2009). T follicular helper cells differentiate from Th2 cells in response to helminth antigens. *J Exp Med, 206*(5), 991-999. doi:10.1084/jem.20090303

Hara, T., Shitara, S., Imai, K., Miyachi, H., Kitano, S., Yao, H., . . . Ikuta, K. (2012). Identification of IL-7-producing cells in primary and secondary lymphoid organs using IL-7-GFP knock-in mice. *J Immunol, 189*(4), 1577-1584. doi:10.4049/jimmunol.1200586

Heng, T. S., Painter, M. W., & Immunological Genome Project, C. (2008). The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol, 9*(10), 1091-1094. doi:10.1038/ni1008-1091

Junt, T., Scandella, E., & Ludewig, B. (2008). Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. *Nat Rev Immunol, 8*(10), 764-775. doi:10.1038/nri2414

Kain, M. J., & Owens, B. M. (2013). Stromal cell regulation of homeostatic and inflammatory lymphoid organogenesis. *Immunology, 140*(1), 12-21. doi:10.1111/imm.12119

Kammer, W., Lischke, A., Moriggl, R., Groner, B., Ziemiecki, A., Gurniak, C. B., . . . Friedrich, K. (1996). Homodimerization of interleukin-4 receptor alpha chain can induce intracellular signaling. *J Biol Chem, 271*(39), 23634-23637.

Katakai, T., & Kinashi, T. (2016). Microenvironmental Control of High-Speed Interstitial T Cell Migration in the Lymph Node. *Front Immunol, 7*, 194. doi:10.3389/fimmu.2016.00194

Kedl, R. M., Lindsay, R. S., Finlon, J. M., Lucas, E. D., Friedman, R. S., & Tamburini, B. A. J. (2017). Migratory dendritic cells acquire and present lymphatic endothelial cell-archived antigens during lymph node contraction. *Nat Commun, 8*(1), 2034. doi:10.1038/s41467-017-02247-z

Klaus, G. G., Humphrey, J. H., Kunkl, A., & Dongworth, D. W. (1980). The follicular dendritic cell: its role in antigen presentation in the generation of immunological memory. *Immunol Rev, 53*, 3-28.

Knowles, D. W., Sudar, D., Bator-Kelly, C., Bissell, M. J., & Lelievre, S. A. (2006). Automated local bright feature image analysis of nuclear protein distribution identifies changes in tissue phenotype. *Proc Natl Acad Sci U S A, 103*(12), 4445-4450. doi:10.1073/pnas.0509944102

Koni, P. A., Sacca, R., Lawton, P., Browning, J. L., Ruddle, N. H., & Flavell, R. A. (1997). Distinct roles in lymphoid organogenesis for lymphotoxins alpha and beta revealed in lymphotoxin beta-deficient mice. *Immunity, 6*(4), 491-500.

Kumar, V., Dasoveanu, D. C., Chyou, S., Tzeng, T. C., Rozo, C., Liang, Y., . . . Lu, T. T. (2015). A dendritic-cell-stromal axis maintains immune responses in lymph nodes. *Immunity, 42*(4), 719-730. doi:10.1016/j.immuni.2015.03.015

Liang, H. E., Reinhardt, R. L., Bando, J. K., Sullivan, B. M., Ho, I. C., & Locksley, R. M. (2011). Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat Immunol, 13*(1), 58-66. doi:10.1038/ni.2182

Lindblad, E. B. (2004). Aluminium adjuvants--in retrospect and prospect. *Vaccine, 22*(27-28), 3658-3668. doi:10.1016/j.vaccine.2004.03.032

Luther, S. A., Tang, H. L., Hyman, P. L., Farr, A. G., & Cyster, J. G. (2000). Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proc Natl Acad Sci U S A, 97*(23), 12694-12699. doi:10.1073/pnas.97.23.12694

Luzina, I. G., Keegan, A. D., Heller, N. M., Rook, G. A., Shea-Donohue, T., & Atamas, S. P. (2012). Regulation of inflammation by interleukin-4: a review of "alternatives". *J Leukoc Biol, 92*(4), 753-764. doi:10.1189/jlb.0412214

MacDonald, A. S., Patton, E. A., La Flamme, A. C., Araujo, M. I., Huxtable, C. R., Bauman, B., & Pearce, E. J. (2002). Impaired Th2 development and increased mortality during Schistosoma mansoni infection in the absence of CD40/CD154 interaction. *J Immunol, 168*(9), 4643-4649.

Mackay, F., Majeau, G. R., Lawton, P., Hochman, P. S., & Browning, J. L. (1997). Lymphotoxin but not tumor necrosis factor functions to maintain splenic architecture and humoral responsiveness in adult mice. *Eur J Immunol, 27*(8), 2033-2042. doi:10.1002/eji.1830270830

McCarthy, D. D., Summers-Deluca, L., Vu, F., Chiu, S., Gao, Y., & Gommerman, J. L. (2006). The lymphotoxin pathway: beyond lymph node development. *Immunol Res, 35*(1-2), 41-54. doi:10.1385/IR:35:1:41

Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., & Hill, A. M. (2000). M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol, 164*(12), 6166-6173.

Mosmann, T. R., Bond, M. W., Coffman, R. L., Ohara, J., & Paul, W. E. (1986). T-cell and mast cell lines respond to B-cell stimulatory factor 1. *Proc Natl Acad Sci U S A, 83*(15), 5654-5658.

Moussion, C., & Girard, J. P. (2011). Dendritic cells control lymphocyte entry to lymph nodes through high endothelial venules. *Nature, 479*(7374), 542-546. doi:10.1038/nature10540

Mueller, S. N., & Germain, R. N. (2009). Stromal cell contributions to the homeostasis and functionality of the immune system. *Nat Rev Immunol, 9*(9), 618-629. doi:10.1038/nri2588

Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J., & Paul, W. E. (1999). The IL-4 receptor: signaling mechanisms and biologic functions. *Annu Rev Immunol, 17*, 701-738. doi:10.1146/annurev.immunol.17.1.701

Ng, C. T., Nayak, B. P., Schmedt, C., & Oldstone, M. B. (2012). Immortalized clones of fibroblastic reticular cells activate virus-specific T cells during virus infection. *Proc Natl Acad Sci U S A, 109*(20), 7823-7828. doi:10.1073/pnas.1205850109

Ngo, V. N., Korner, H., Gunn, M. D., Schmidt, K. N., Riminton, D. S., Cooper, M. D., . . . Cyster, J. G. (1999). Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med, 189*(2), 403-412.

Onder, L., Danuser, R., Scandella, E., Firner, S., Chai, Q., Hehlgans, T., . . . Ludewig, B. (2013). Endothelial cell-specific lymphotoxin-beta receptor signaling is critical for lymph node and high endothelial venule formation. *J Exp Med, 210*(3), 465-473. doi:10.1084/jem.20121462

Onder, L., Morbe, U., Pikor, N., Novkovic, M., Cheng, H. W., Hehlgans, T., . . . Ludewig, B. (2017). Lymphatic Endothelial Cells Control Initiation of Lymph Node Organogenesis. *Immunity, 47*(1), 80-92 e84. doi:10.1016/j.immuni.2017.05.008

Onder, L., Narang, P., Scandella, E., Chai, Q., Iolyeva, M., Hoorweg, K., . . . Ludewig, B. (2012). IL-7-producing stromal cells are critical for lymph node remodeling. *Blood, 120*(24), 4675-4683. doi:10.1182/blood-2012-03-416859

Pandey, S., Mourcin, F., Marchand, T., Nayar, S., Guirriec, M., Pangault, C., . . . Tarte, K. (2017). IL-4/CXCL12 loop is a key regulator of lymphoid stroma function in follicular lymphoma. *Blood, 129*(18), 2507-2518. doi:10.1182/blood-2016-08-737239

Paul, W. E. (1991). Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood, 77*(9), 1859-1870.

Pelly, V. S., Kannan, Y., Coomes, S. M., Entwistle, L. J., Ruckerl, D., Seddon, B., . . . Wilson, M. S. (2016). IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection. *Mucosal Immunol, 9*(6), 1407-1417. doi:10.1038/mi.2016.4

Reynes, M., Aubert, J. P., Cohen, J. H., Audouin, J., Tricottet, V., Diebold, J., & Kazatchkine, M. D. (1985). Human follicular dendritic cells express CR1, CR2, and CR3 complement receptor antigens. *J Immunol, 135*(4), 2687-2694.

Round, J. L., & Mazmanian, S. K. (2009). The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol, 9*(5), 313-323. doi:10.1038/nri2515

Ruddle, N. H., & Akirav, E. M. (2009). Secondary lymphoid organs: responding to genetic and environmental cues in ontogeny and the immune response. *J Immunol, 183*(4), 2205-2212. doi:10.4049/jimmunol.0804324

Sellers, R. S., Clifford, C. B., Treuting, P. M., & Brayton, C. (2012). Immunological variation between inbred laboratory mouse strains: points to consider in phenotyping genetically immunomodified mice. *Vet Pathol, 49*(1), 32-43. doi:10.1177/0300985811429314

Stetson, D. B., Mohrs, M., Reinhardt, R. L., Baron, J. L., Wang, Z. E., Gapin, L., . . . Locksley, R. M. (2003). Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med, 198*(7), 1069-1076. doi:10.1084/jem.20030630

Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., . . . Akira, S. (1996). Essential role of Stat6 in IL-4 signalling. *Nature, 380*(6575), 627-630. doi:10.1038/380627a0

Tamburini, B. A., Burchill, M. A., & Kedl, R. M. (2014). Antigen capture and archiving by lymphatic endothelial cells following vaccination or viral infection. *Nat Commun, 5*, 3989. doi:10.1038/ncomms4989

Taylor, J. J., Krawczyk, C. M., Mohrs, M., & Pearce, E. J. (2009). Th2 cell hyporesponsiveness during chronic murine schistosomiasis is cell intrinsic and linked to GRAIL expression. *J Clin Invest, 119*(4), 1019-1028. doi:10.1172/JCI36534

Thomas, S. N., Rutkowski, J. M., Pasquier, M., Kuan, E. L., Alitalo, K., Randolph, G. J., & Swartz, M. A. (2012). Impaired humoral immunity and tolerance in K14-VEGFR-3-Ig mice that lack dermal lymphatic drainage. *J Immunol, 189*(5), 2181-2190. doi:10.4049/jimmunol.1103545

van Buul, J. D., Kanters, E., & Hordijk, P. L. (2007). Endothelial signaling by Ig-like cell adhesion molecules. *Arterioscler Thromb Vasc Biol, 27*(9), 1870-1876. doi:10.1161/ATVBAHA.107.145821

Vitetta, E. S., Ohara, J., Myers, C. D., Layton, J. E., Krammer, P. H., & Paul, W. E. (1985). Serological, biochemical, and functional identity of B cell-stimulatory factor 1 and B cell differentiation factor for IgG1. *J Exp Med, 162*(5), 1726-1731.

Yeo, K. P., & Angeli, V. (2017). Bidirectional Crosstalk between Lymphatic Endothelial Cell and T Cell and Its Implications in Tumor Immunity. *Front Immunol, 8*, 83. doi:10.3389/fimmu.2017.00083

Zhou, Y. W., Aritake, S., Tri Endharti, A., Wu, J., Hayakawa, A., Nakashima, I., & Suzuki, H. (2003). Murine lymph node-derived stromal cells effectively support survival but induce no activation/proliferation of peripheral resting T cells in vitro. *Immunology, 109*(4), 496-503.

Zindl, C. L., Kim, T. H., Zeng, M., Archambault, A. S., Grayson, M. H., Choi, K., . . . Chaplin, D. D. (2009). The lymphotoxin LTalpha(1)beta(2) controls postnatal and adult spleen marginal sinus vascular structure and function. *Immunity, 30*(3), 408-420. doi:10.1016/j.immuni.2009.01.010

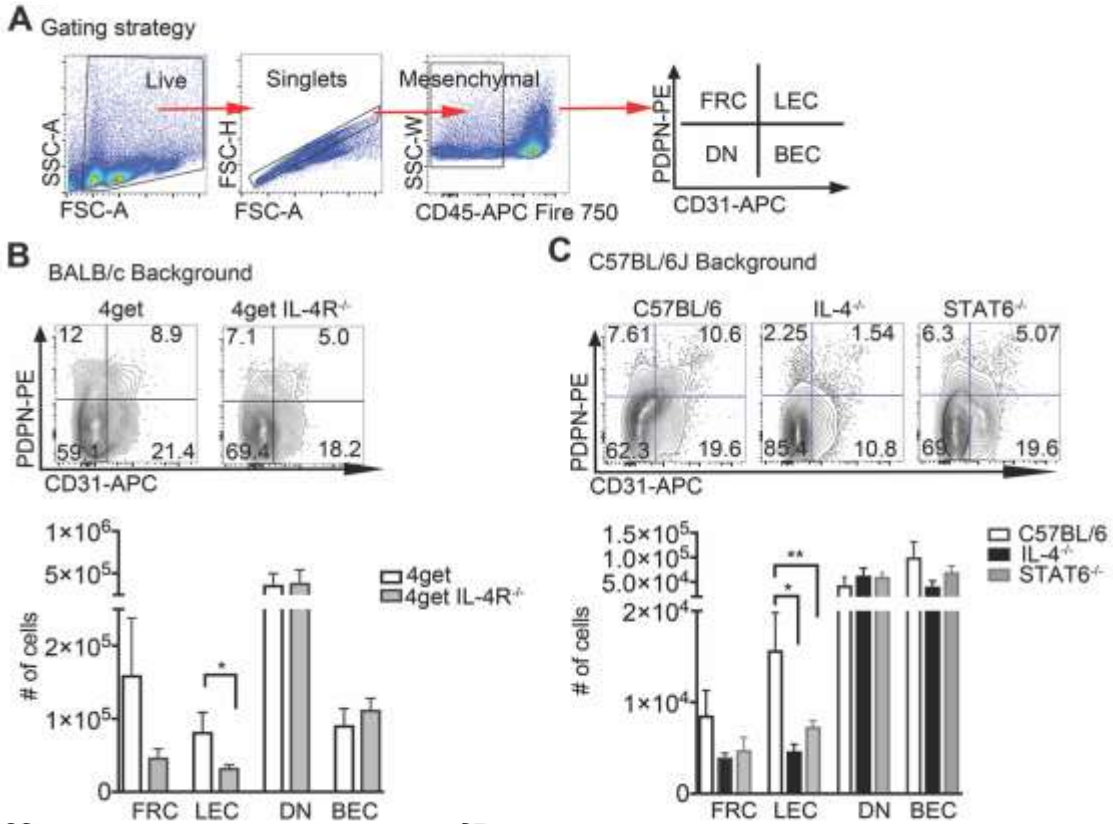

Figure 2.1 IL-4 deficiency in peripheral lymph nodes correlates to decreased cellularity of lymphatic endothelial cells

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Popliteal lymph nodes from naïve BALB/c and C57BL/6 mice were collected, digested with collagenase D/ DNAse I and single cells suspensions were analyzed for flow cytometry. (A) Gating strategy for Fibroblastic reticular cells (FRC), Lymphatic endothelial cells (LEC), Blood Endothelial Cells (BEC) and Double Negatives (DN). (B) Flow cytometry analysis and cell numbers in a BALB/c mice (C) Flow cytometry analysis and cell numbers in a C57BL/6 background. Student t-test was used to determine statistical significance *$p < 0.05$, **$p < 0.01$. FACS data shown are concatenated from 3-5 mice per group and experiments were performed 3-4 times.

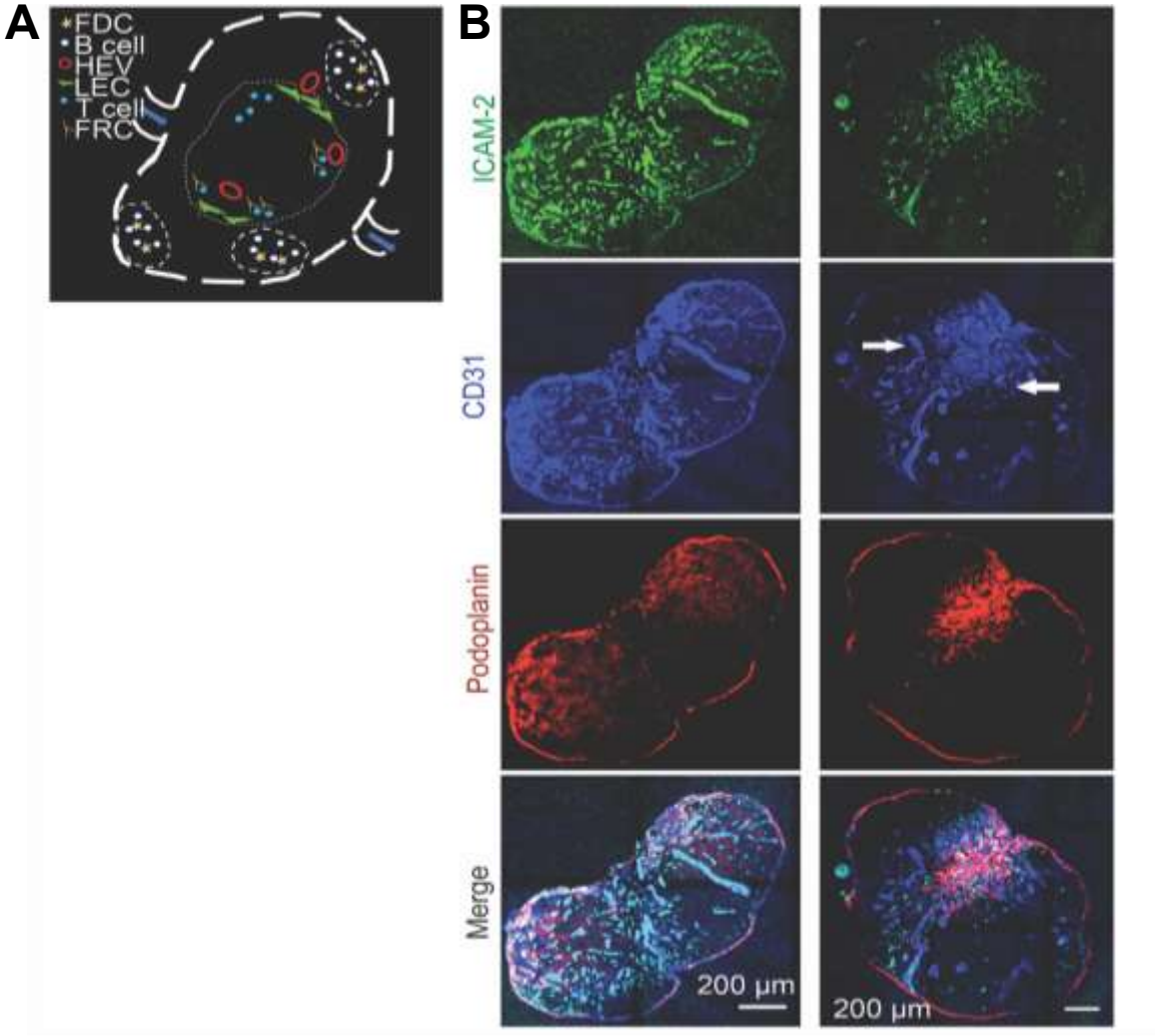


Figure 2.2 Maintenance of endothelial cell organization in peripheral lymph nodes in steady state requires IL-4 signaling

Popliteal lymph nodes from naïve BALB/c and C57BL/6 mice were collected in OCT, frozen and sliced in 10 micron for confocal microscopy analysis (A) Schematic of lymph node showing cells of interest in B cell follicles and T cell area (B) Popliteal lymph node cryosections were stained for ICAM-2, green; CD31, blue and podoplanin, red. Scale bar: 200 µm. Arrowhead denotes CD31+ endothelial cells clusters preferentially localized in the medulla. Confocal images are representative of three different experiments with 3 mice per group.

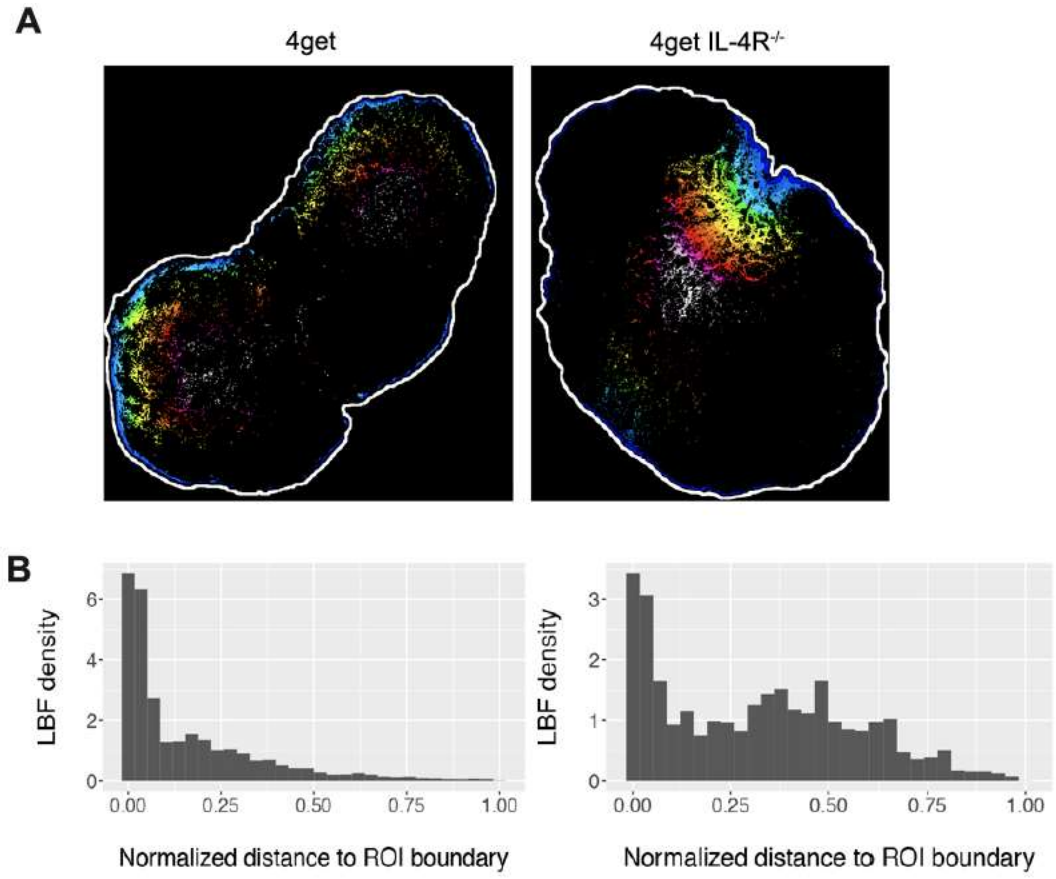


Figure 2.3 Visualization of differences in spatial distribution of co-stained podoplanin/CD31 locally bright features (LBF).

The LBF color encodes the distance to the Region of Interest (ROI) boundary (white). (A)The LBFs in the lymph node located close to the perimeter of the ROI (shown as blue, cyan and green) and the proportion of LBF which are away from the ROI boundary (encoded in yellow, red and magenta) is shown. (B) Quantification of the LBF density to the normalized distance to the ROI boundary. Image is representative of >3 mice from two independent experiments.

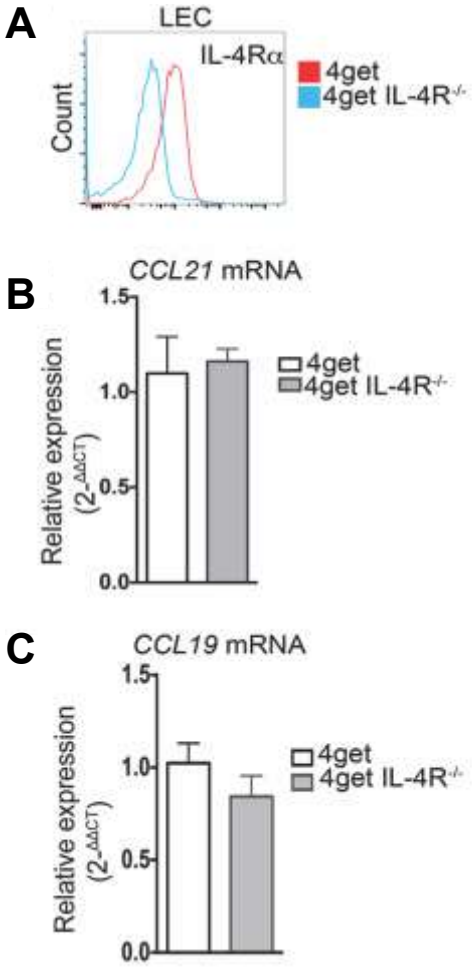


Figure 2.4 IL-4 mediated defect in endothelial cell is not dependent on CCL19 or CCL21.

Lymph nodes in steady state requires IL-4 signaling. (A) Expression of IL-4Rα in lymphatic endothelial cells from popliteal lymph node determined by flow cytometry (B) Relative expression of CCL21 (C) Relative expression of CCL19 by quantitative PCR, respectively from naïve popliteal lymph nodes normalized to naïve 4get (control), bars show mean ± SEM.

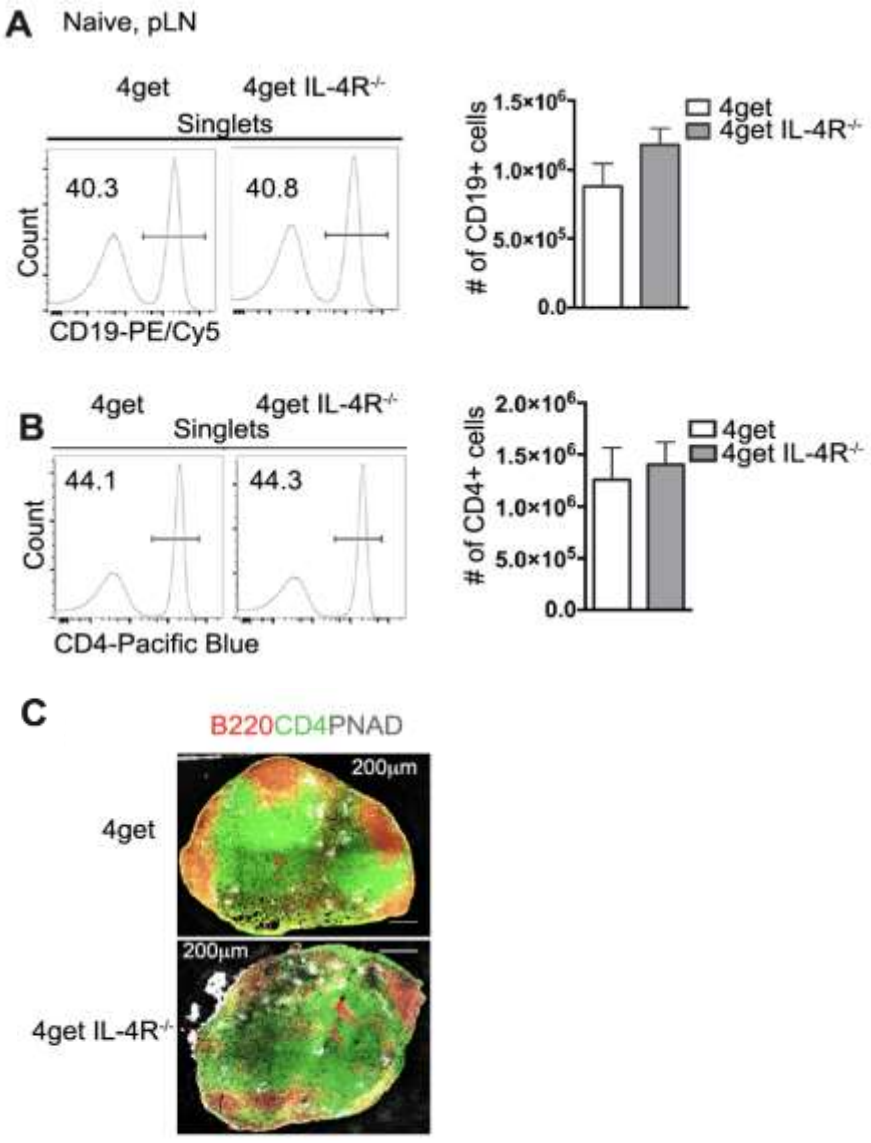


Figure 2.5 Lack of IL-4Rα does not affect T and B cell numbers or high endothelial venules in naive peripheral lymph nodes in BALB/c background.

(A) Frequency and number of B cells by flow cytometry in WT (4get) and 4get IL-4RKO. (B) Frequency and number of T cells (C) Tile confocal imaging of naive pLN stained with B220 (red), CD4 (green) and PNAd high endothelial venules (HEV)(gray). Scale bar: 200 μm. One representative image from three independent experiments is shown. FACS data depicts concatenated samples from >3 mice per group from four independent experiments. Bars show mean ±SEM from four independent experiments.

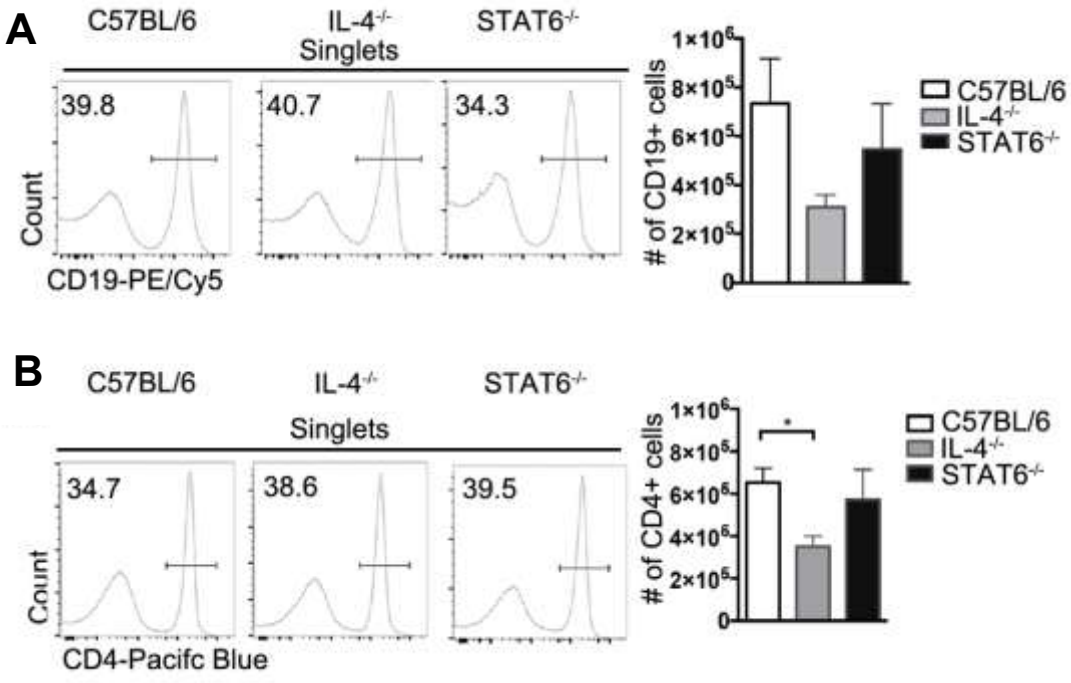


Figure 2.6 Lack of IL-4Rα does not affect T and B cell numbers or high endothelial venules in naive peripheral lymph nodes in a C57BL/6 background.

(A) CD19+ expression and B cell numbers in WT, IL-4KOand STAT6KO (B) Frequency and number of CD4+ T cells in naive popliteal lymph node of WT, IL-4KO and STAT6KO. FACS data depicts concatenated samples from >3 mice per group from four independent experiments. Bars show mean ±SEM from four independent experiments. Student's t-test was used to determine statistical significance, *$p < 0.05$.

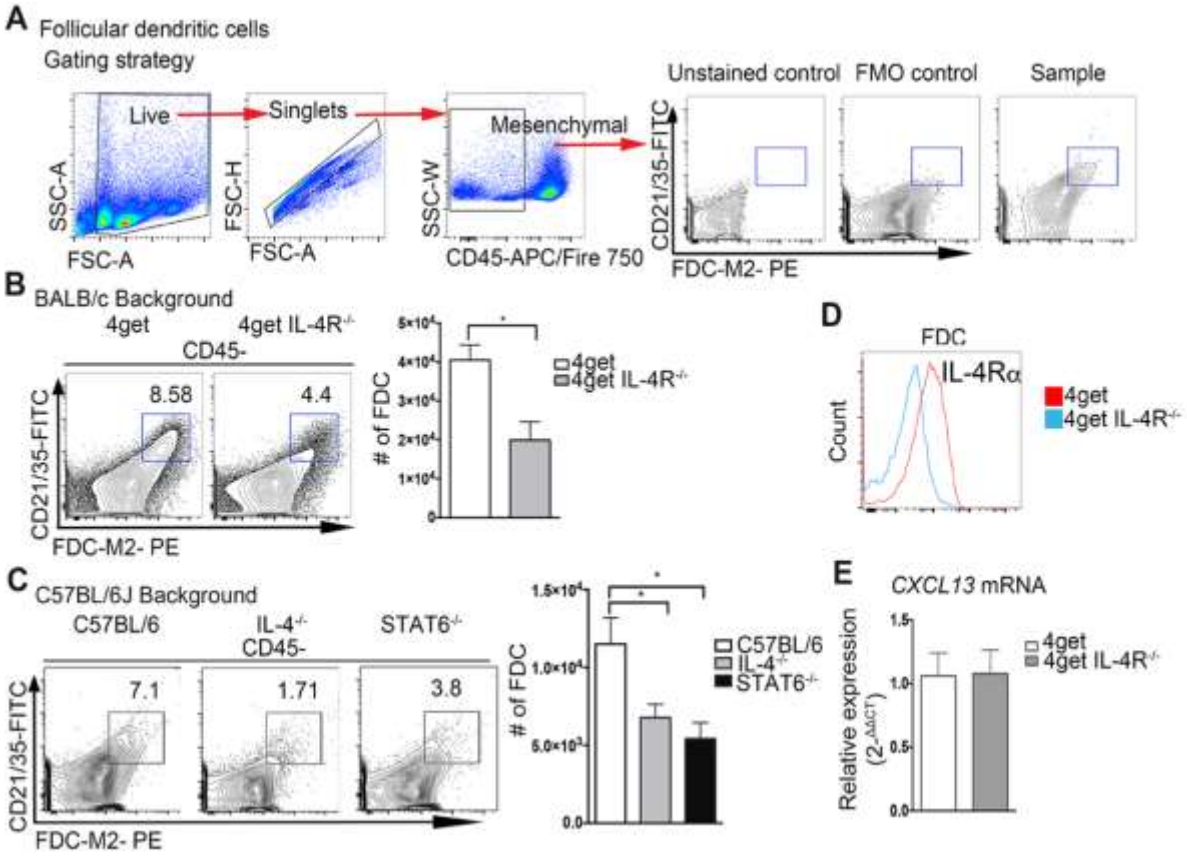


Figure 2.7 IL-4 is required for maintenance of follicular dendritic cell in peripheral lymph nodes in steady state. .

Lymph nodes from naïve C57BL/6J, IL-4KO, STAT6KO, 4get homozygous and 4get IL-4RαKO mice were digested with collagenase and smashed through a cell strainer to be analyzed by flow cytometry or frozen in OCT medium, and tile scans of cryosections were acquired with a laser scanning confocal microscope. (A) Gating strategy and staining controls. (B) Follicular dendritic cells (CD21/35^{+} FDC-M2^{+}) gated from CD45^{-} obtained from 4get homozygous and 4get IL-4Rα KO popliteal lymph nodes and (C) popliteal lymph nodes of C57BL/6J, IL-4KO, STAT6KO mice. (D) Expression of IL-4Rα in follicular dendritic cells from naïve popliteal lymph node. (E) Relative expression of CXCL13 from naïve pLNs, normalized to naïve 4get (control) bars show mean ± SEM.

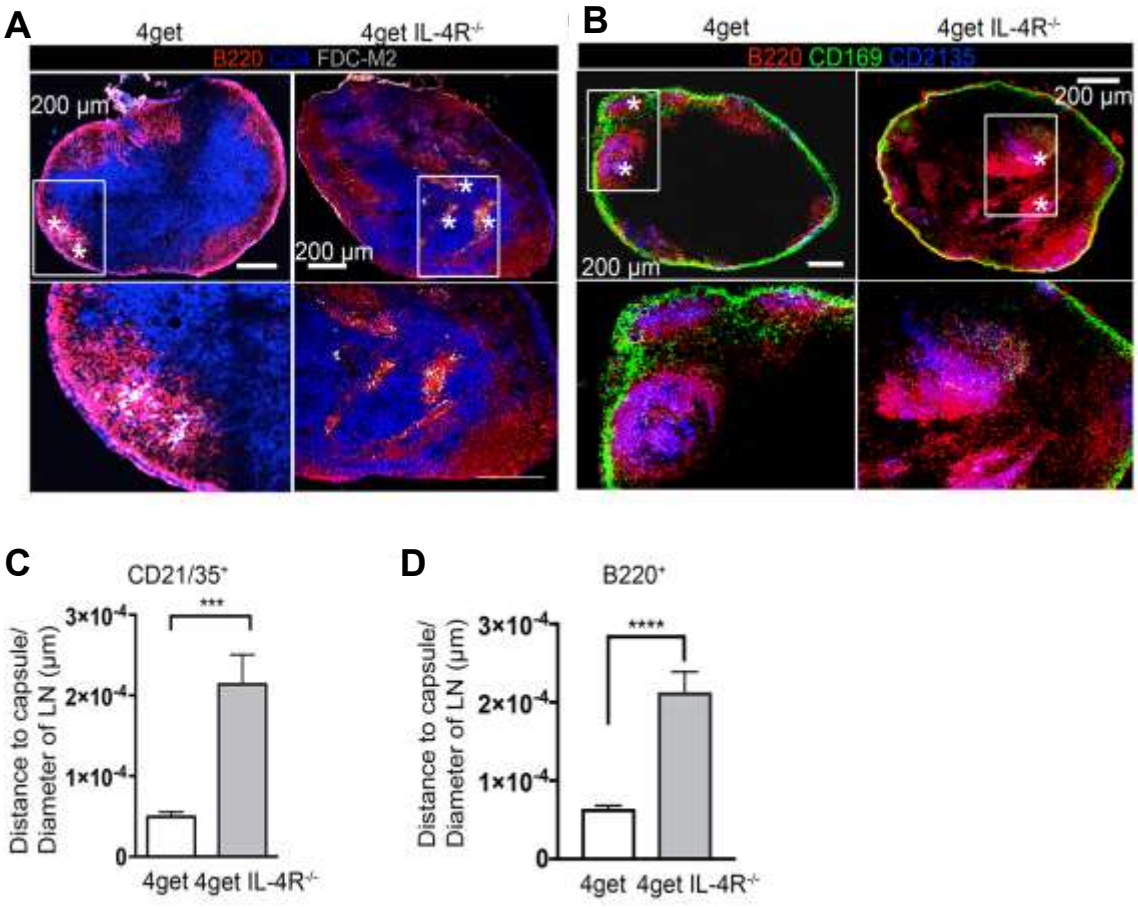


Figure 2.8 IL-4 is required for proper follicular dendritic cell and B cell follicle organization in peripheral lymph nodes in steady state.

(A) Frozen popliteal lymph node sections from 4get homozygous (control) and 4get IL-4RαKO mice were stained for B220 (red), CD4 (blue) and FDC-M2 (gray) (B) Sections were stained for CD21/35 (blue, follicular dendritic cells), B220 (red, B cells) and CD169 (green, subcapsular macrophages). Asterisk denotes FDC-M2$^+$ cells and CD21/35$^+$ cells, scale bar: 200 µm. (C) Quantification of the distance of CD21/35$^+$ FDC to the LN capsule normalized to the LN diameter. (D) Quantification of B220$^+$ cells from the lymph node capsule and normalized to the total lymph node area. Bar depicts mean ± SEM.

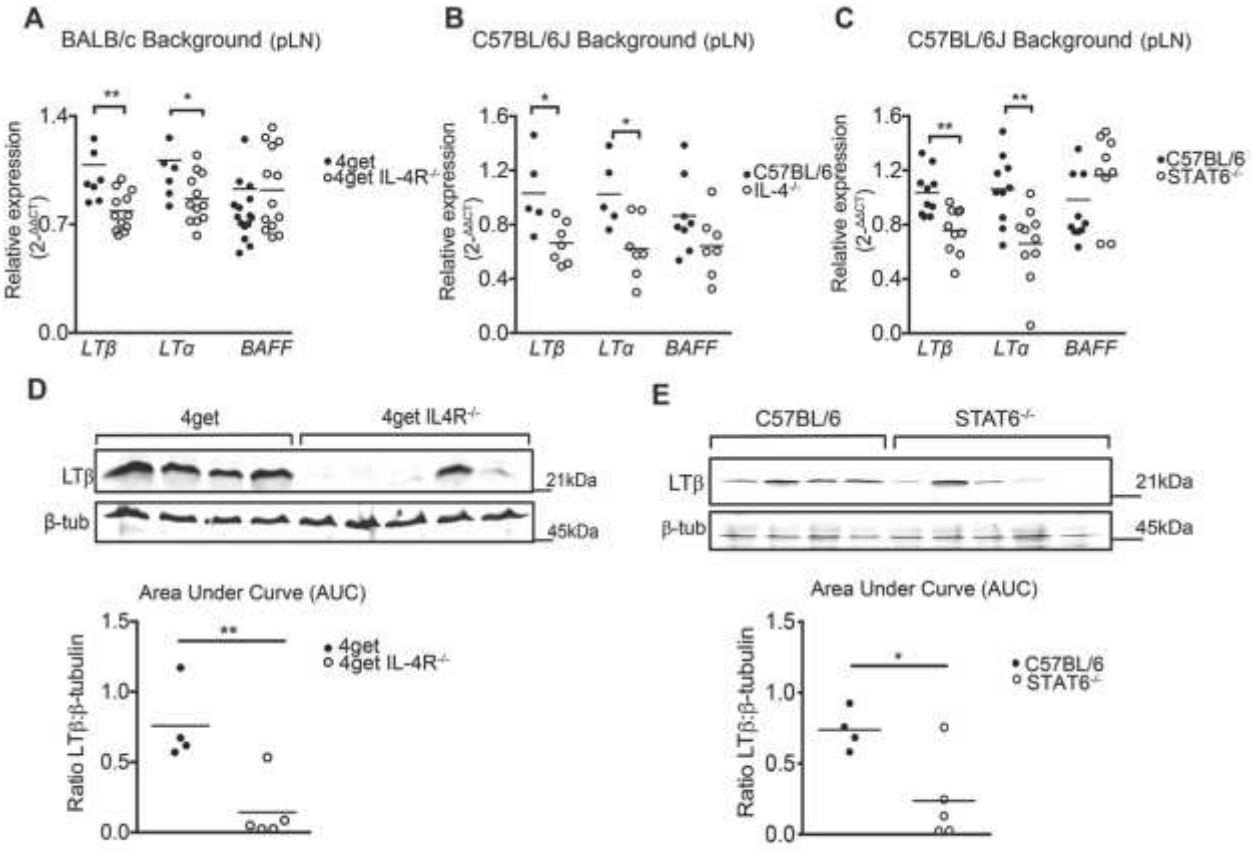


Figure 2.9 LTβ and LTα mRNA levels in peripheral lymph nodes are dependent on the IL-4 signaling pathway in steady state.

4get homozygous and 4get IL-4RαKO, as well as C57BL/6J and IL-4KO and STAT6KO mice were sacrificed and popliteal lymph nodes were collected. (A) Relative expression of LTβ, LTα and BAFF normalized to beta-actin in 4get homozygous and 4get IL-4RKO mice. (B) Relative expression of LTα and LTβ and BAFF in whole popliteal lymph nodes from C57BL/6J and STAT6-/- and (C) C57BL/6J and IL-4KO mice. (D) LTβ protein expression normalized to β-tubulin in WT and 4get IL4RαKO mice. (E) LTβ protein expression normalized to β-tubulin in WT and STAT6KO mice. Statistical significance was determined by Man-Whitney test *$p < 0.05$, **$p < 0.01$. Data points represent individual mice. Quantitative PCR data represents three different experiments with n>4 mice/group. Western blot data are representative of two independent experiments with n≥4 mice per group.

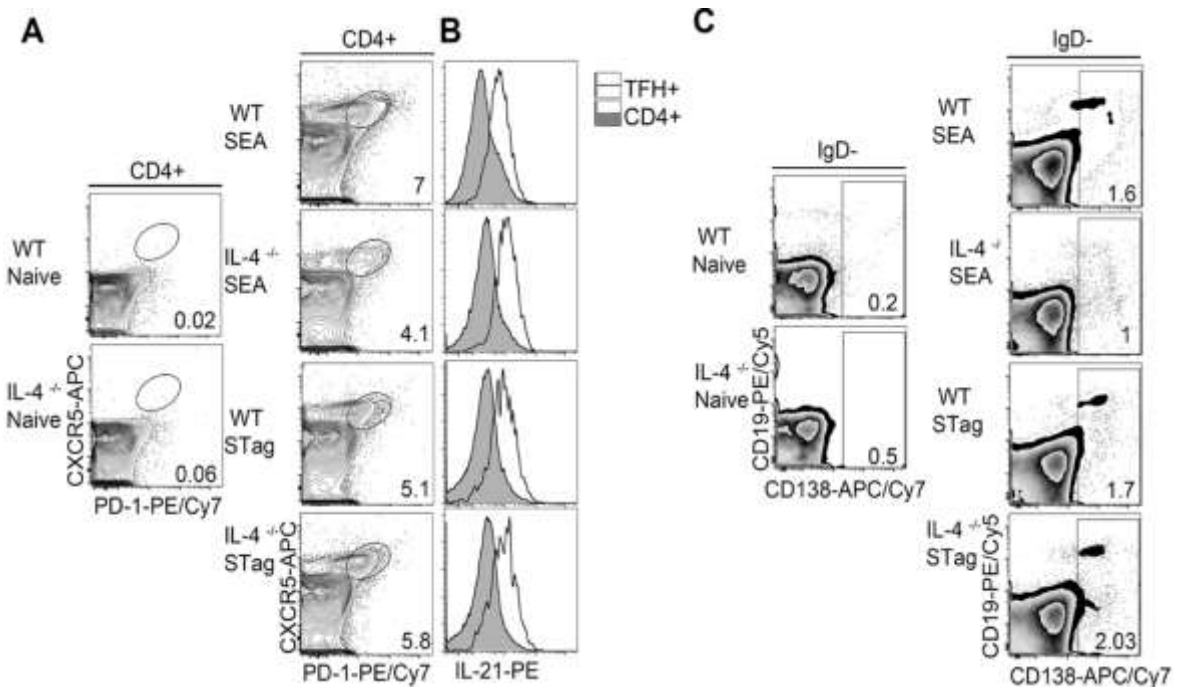


Figure 2.10 IL-4 is required for lymph node re-organization and germinal center formation following immunization with SEA, but not STag antigens.

C57BL/6J and IL-4KO mice were immunized s.c. in the footpad with either SEA or STag and draining and non-draining lymph nodes collected 8-days post-immunization. Lymph nodes were used to obtain single cell suspension for flow cytometry or frozen in OCT medium and section for imaging by confocal microscopy. (A) Frequency of T follicular helper cells ($CD4^+$ $CXCR5^+PD\text{-}1^+$) in WT mice and IL-4KO from naïve or immunized with either SEA or STag. (B) Expression of IL-21 in TFH^+ and $CD4^+$ cells from WT mice and IL-4KO immunized with either SEA or Stag. (C) Plasma cells ($CD19^{+/-}$ and $CD138^+$) pre-gated on IgD^- cells.

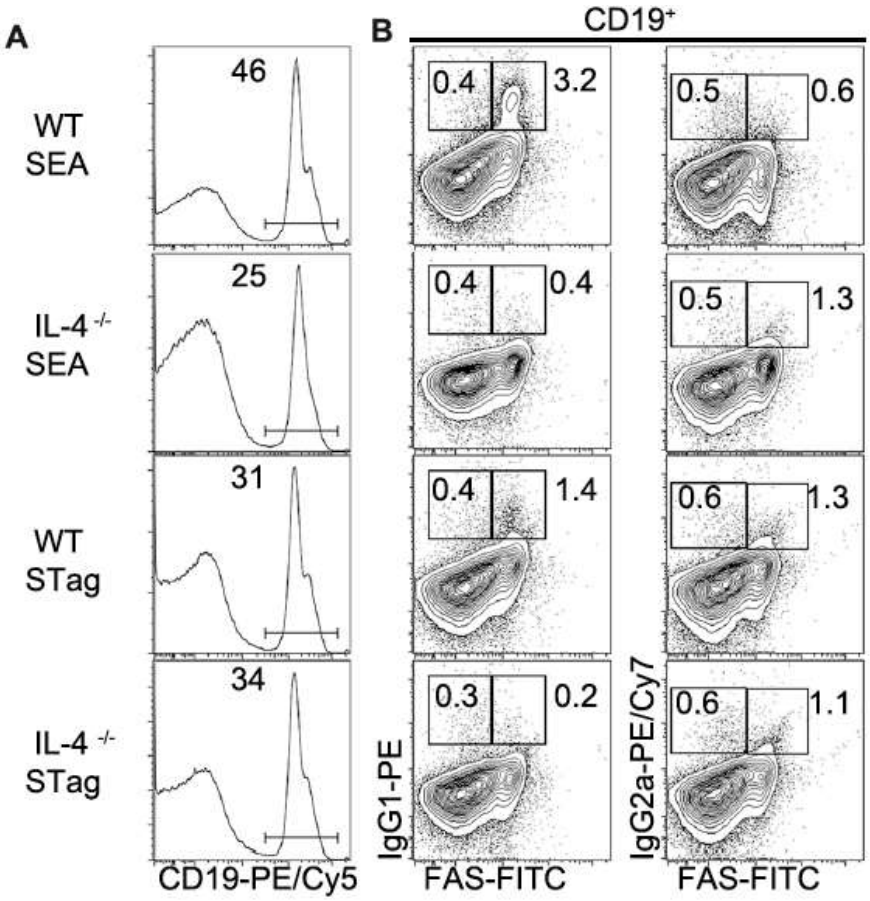


Figure 2.11In absence of IL-4, Type 2, but not Type 1 antigens, fail to induce a humoral response.

C57BL/6 and IL-4KO of 6-8 weeks of age were immunized with 30μg of S. mansoni antigens or T. gondii antigens. 8 days post-immunization, draining popliteal lymph nodes were collected to obtain single cell suspensions and analyzed by flow cytometry. (A) Expression of CD19+ cells (B cells) gated from lymphocytes, (B) Isotype-switched IgG1(FAS+IgG1+ GC B cells or Fas−IgG1+ memory B cells) and IgG2a (GC B cells or memory) from B cells. Plots represent concatenated samples from five independent experiments and >3 mice per group.

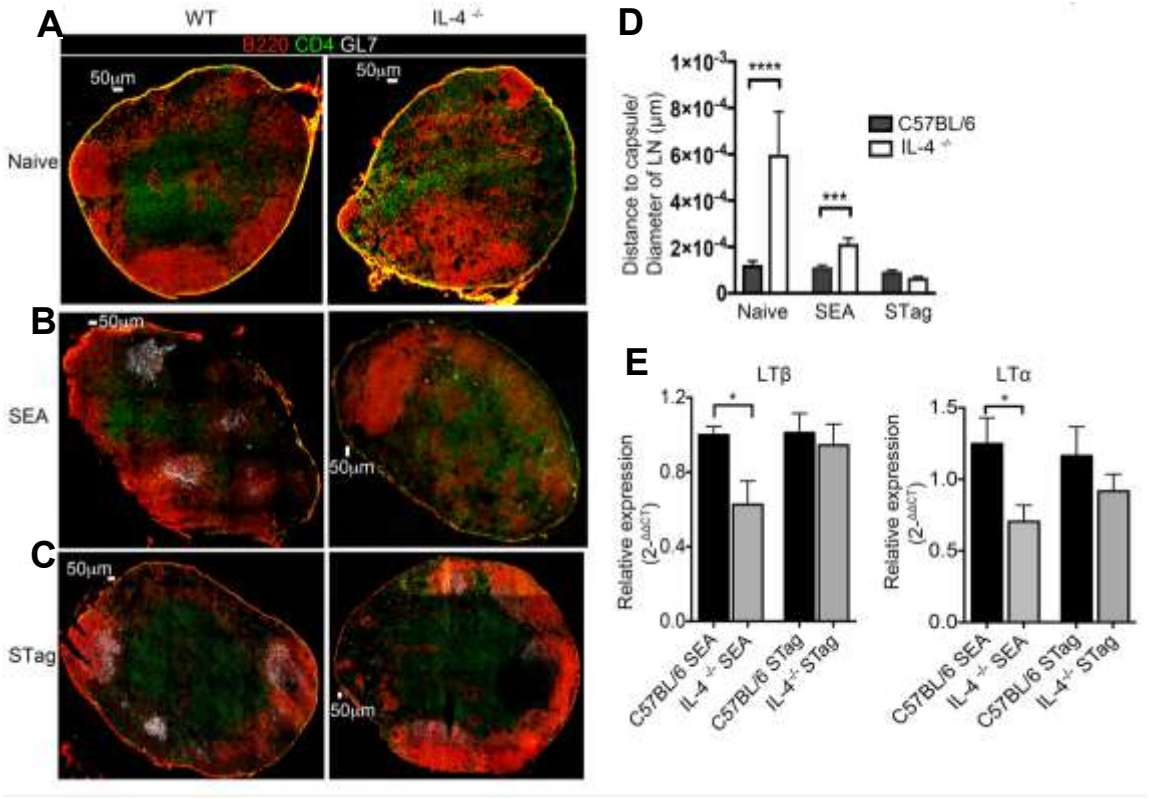


Figure 2.12 Type 1 antigens but not Type 2 antigens can rescue lymph node organization and lymphotoxin expression.

(A) Cryosections of popliteal lymph node of naïve WT (C56BL/6J) and IL-4KO mice stained with B220 (red) GL7 (gray), CD4 (green), scale bar: 50 μm. (B) Frozen sections from SEA immunized WT (C57BL/6J) and IL-4KO mice stained for B220 (red) GL7 (gray), CD4 (green). (C) Frozen sections of popliteal lymph nodes from STag immunized C57BL/6J and IL-4KO mice stained for B220 (red) GL7 (gray), CD4 (green). Scale bar: 50 μm. (D) Quantitation of the distance of $B220^{+}$ expression to the capsule of lymph nodes normalized to lymph node area, bar show mean ± SEM. Data representative from three to five mice per group (E) Relative gene expression of LTβ and LTα (normalized to β-actin) in SEA and STag immunized mice. FACS data depict concatenated samples and confocal images shown are representative of 3-5 mice. Data is representative of four independent experiments. Unpaired Student's t test was used to determine statistical significance; *$p<0.05$.

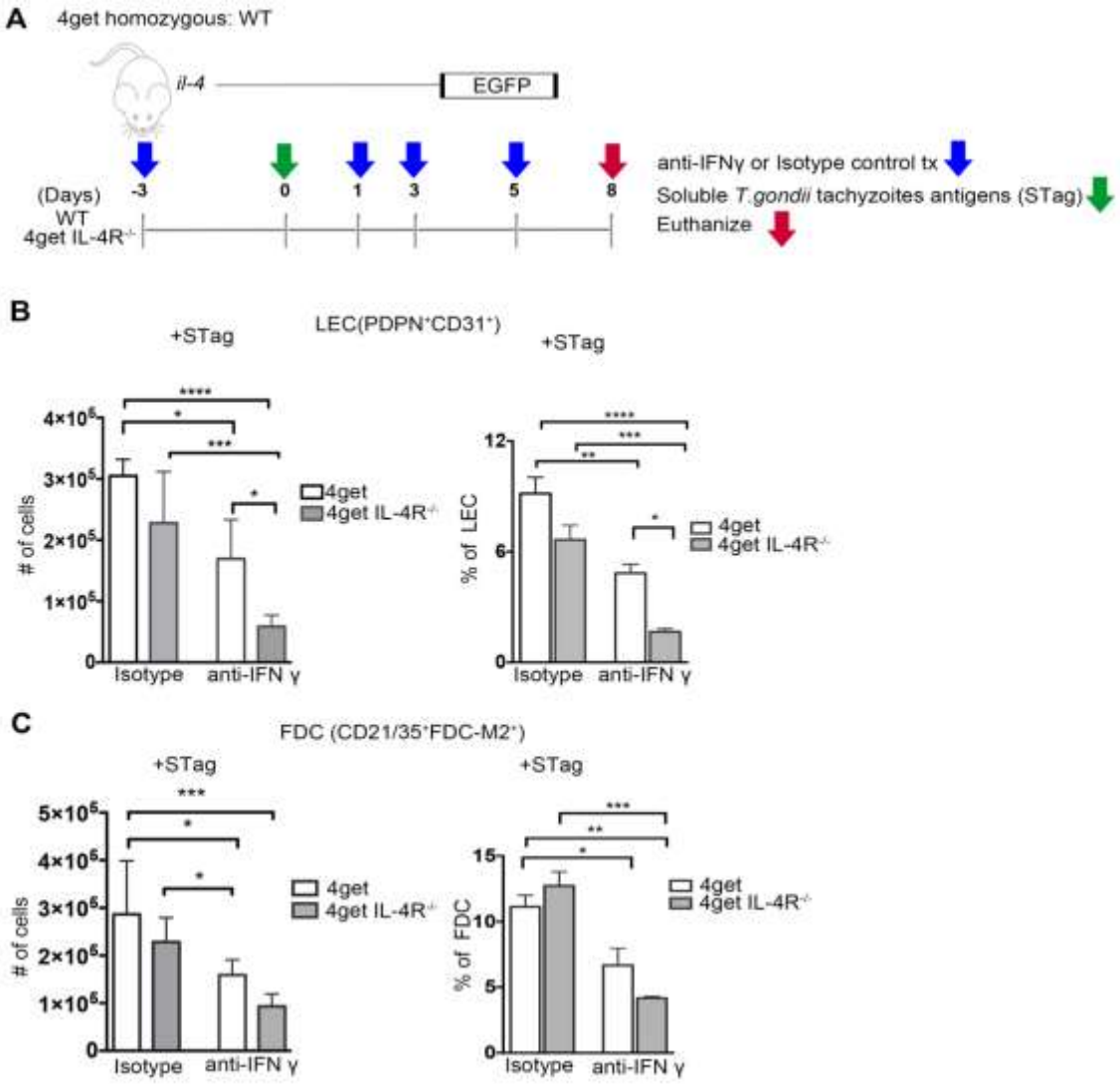


Figure 2.13 IFN-γ promotes Stag induced LEC cell expansion in peripheral lymph nodes in the absence of IL-4Rα.

4get homozygous and 4get IL-4RαKO mice were immunized s.c. in the footpad with STag and treated with either anti-IFNγ or isotype control. (A) Schematic of Stag immunization and blocking antibody treatment. (B) Cell numbers of lymphatic endothelial cells ($PDPN^+CD31^+$) in WT and knockout mice after treatment with isotype control or IFNγ blocking antibody, bars shown mean ± SEM. (C) Number of follicular dendritic cells ($FDC\text{-}M2^+CD21/35^+$) from isotype and IFNγ blocking antibody treated WT and knockout mice were analyzed by flow cytometry. Bars shown mean ± SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$ (ANOVA, Bonferroni's multiple comparison test). FACS data shown are concatenated from 3-6 mice per group, results are from two independent experiments

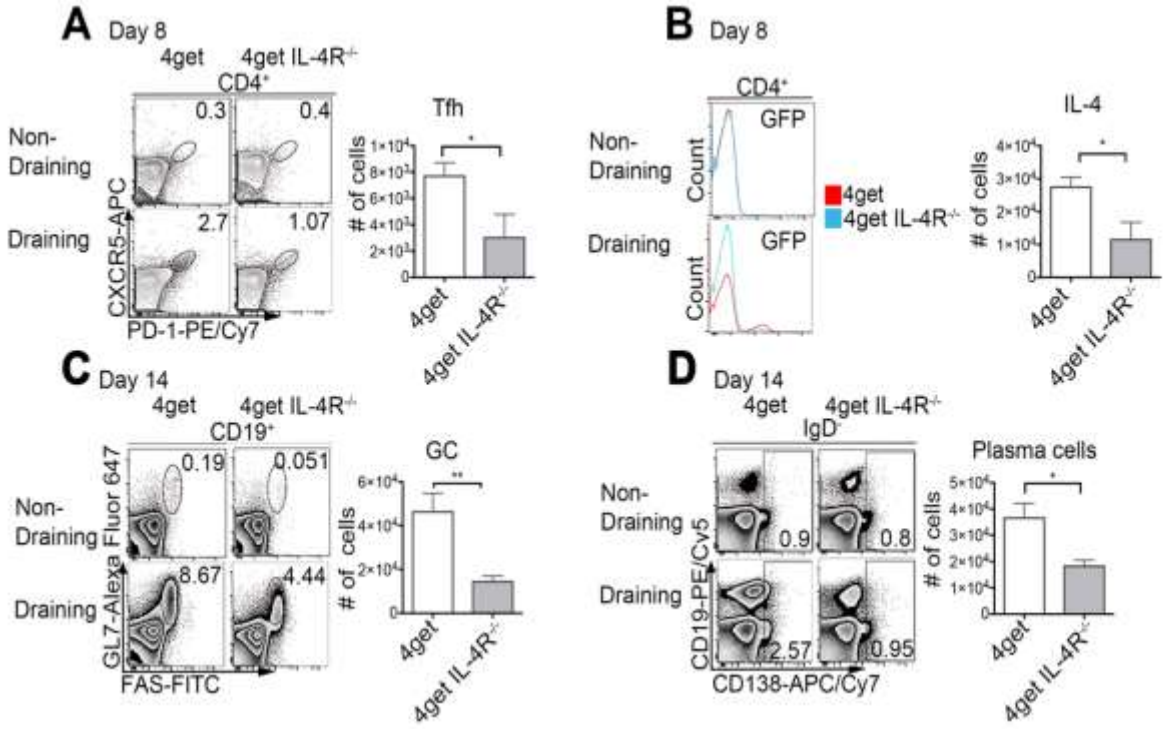


Figure 2.14 Expansion of endothelial cells and follicular dendritic cells is reduced during immunization with Tetanus/Diphtheria (TD) in a IL-4Rα deficient mice.
4get homozygous and 4get IL-4RαKO mice were injected s.c. with 1/10 of the TD (with alum as adjuvant) dose employed in humans. 8 and 14 days post-immunization reactive peripheral lymph nodes were collected, digested and filtered through a cell strainer to obtain a single cell suspension. Cells were stained with surface antibodies and analyzed by flow cytometry. (A) Expression and cell number of T follicular helper cells ($CD4^+CXCR5^+PD\text{-}1^+$) at day 8 from non-draining and reactive popliteal lymph nodes, bar denotes mean ± SEM (B) expression of IL-4 (GFP+) 8 days post-immunization with Tetanus diphtheria (C) Germinal center ($CD19^+GL7^+FAS^+$) at day 14, (D) Plasma cells and plasmablasts ($CD19^{+/-}$ $CD138^+$) at day 14 post-immunization pre-gated on IgD-.

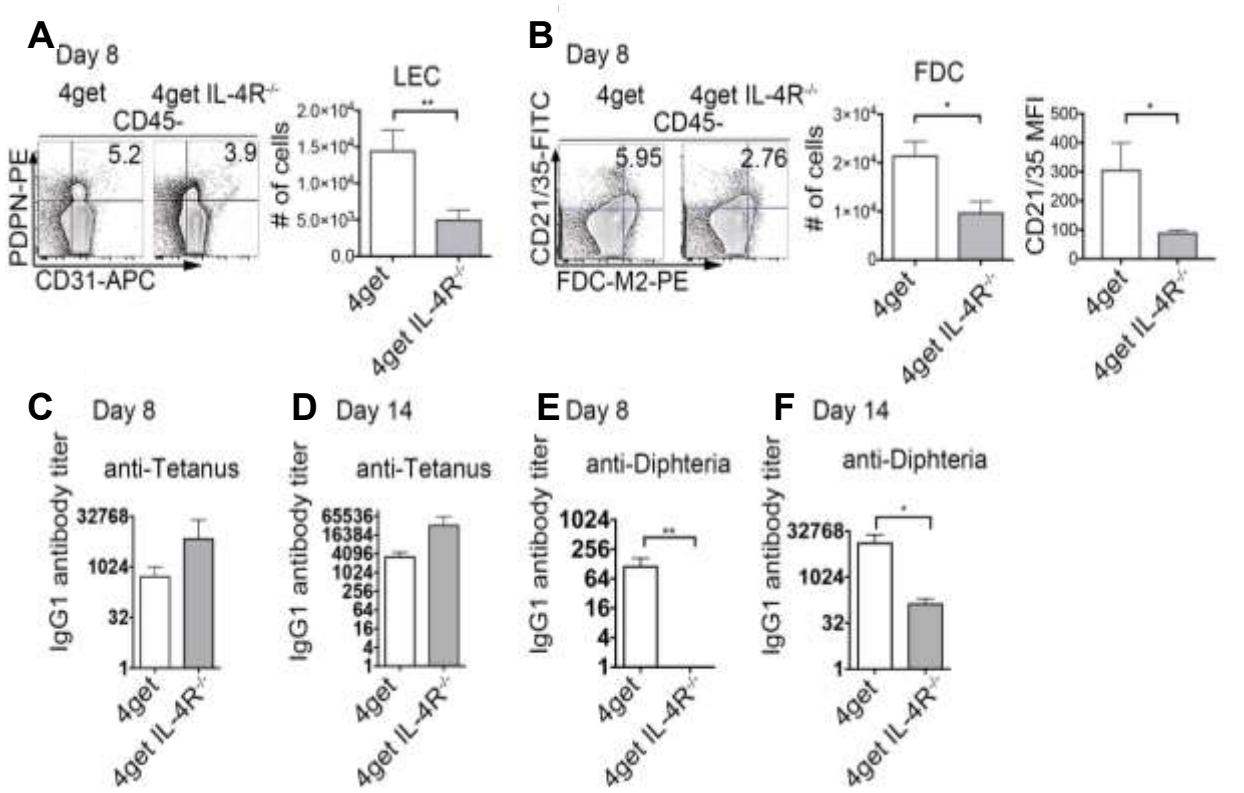


Figure 2.15 Expansion of endothelial cells and follicular dendritic cells is reduced during immunization with Tetanus/Diphtheria (TD) in a IL-4Rα deficient mice.

Immunization with Tetanus/Diphtheria (TD) fails to induce. (A) Lymphatic endothelial cells (PDPN+CD31+) at day 8 and (B) Follicular dendritic cells (FDCM2+CD21/35+) pre-gated on CD45 negative, cell numbers and median fluorescence intensity (MFI), mean ± SEM. (C) Tetanus specific IgG1 antibody titers at days 8 and (D) 14 post immunization. (E) Diphtheria specific IgG1 antibody titers at days 8 and (F) 14 post immunization. FACS data represents concatenated samples from >3 mice per group and representative of three independent experiments for day 14 and two independent experiments for day 8. *$p < 0.05$, **$p < 0.01$; unpaired Student's t-test.

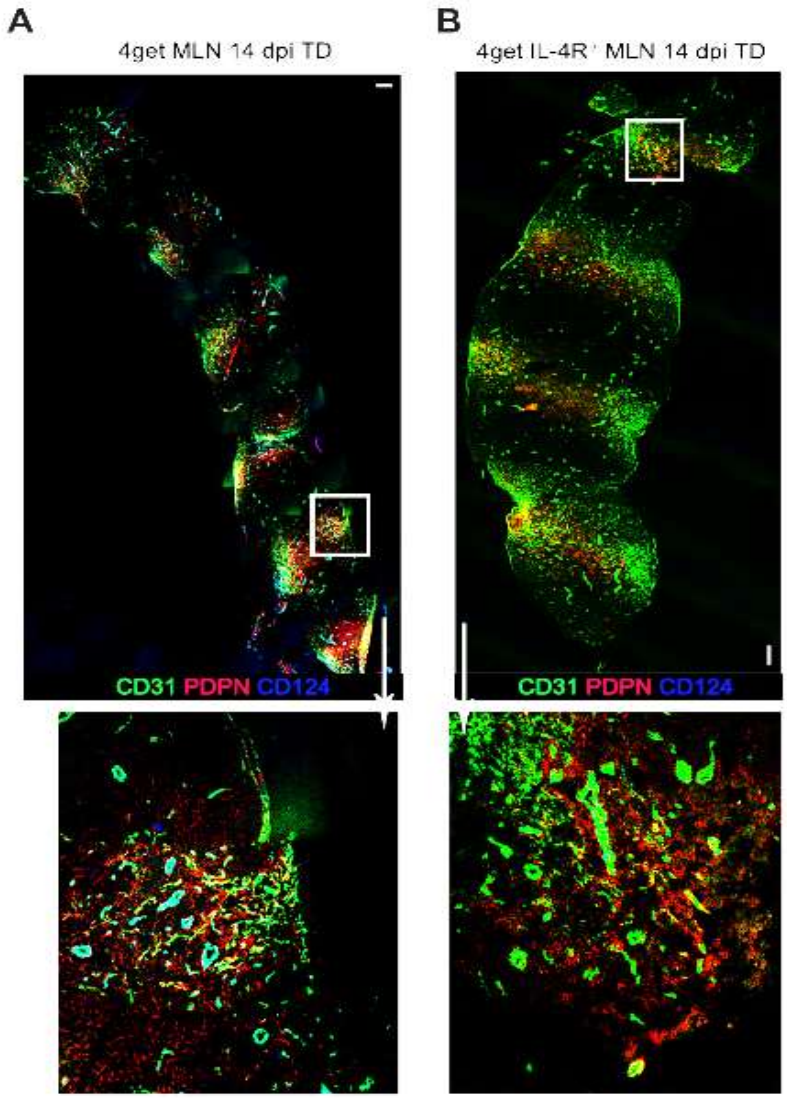


Figure 2.16 Mesenteric lymph node organization following immunization with alum-adjuvanted Tenatus/Diphteria.

Mesenteric lymph nodes from 4get (control) and 4get IL-4Ralpha deficient mice were dissected, collected in OCT as described in the Material and Methods, and frozen. Tile confocal images from 14 days post immunization were obtained and imaged with a Leica TCS SP5 laser scanning confocal microscope (A) Frozen mesenteric lymph nodes from wild type (4get) mice were stained for CD31 (green), podoplanin (PDPN, red) and CD124 (blue). Scale bar: 200 μm.(B) Frozen MLN section from 4get IL-4R alpha stained with CD31(green), PDPN (red) and CD124 (blue) Scale bar: 200 μm. Images are representative of three experiments with three mice per group.

CHAPTER 3. ANTENATAL EXPOSURE TO *S. MANSONI* ANTIGENS INDUCES A STATE OF HYPORESPONSIVENESS IN OFFSPRING

Introduction

Schistosomiasis is an infectious disease caused by trematode parasites of the genus Schistosoma, with *S. mansoni, S. hematobium* and *S. japonicum* causing the most human morbidity (Olveda et al., 2014). The disease affects an estimated 779 million people who are at risk, and 207 million people who are infected annually (Steinmann, Keiser, Bos, Tanner, & Utzinger, 2006). Among these, the highest prevalence occurs in adolescence and early adulthood (10 to 24 years of age), (Colley, Bustinduy, Secor, & King, 2014) with a special impact in women of reproductive age, with 40 millions of them infected. Human studies on schistosomiasis and pregnancy have previously established a link between maternal infection and low birth weight, as well as, premature birth and intrauterine growth restriction(Kurtis et al., 2011; McDonald, Pond-Tor, et al., 2014; Siegrist & Siegrist-Obimpeh, 1992). Moreover, the effects of maternal Schistosomiasis on the response of offspring to heterologous antigens have shown impaired responses in infant immunity at 2 years of age, and older, rising the concern that mother's parasitic status during pregnancy might render early childhood immunization ineffective for years and even decades post-immunization (Ondigo et al., 2018). Thus far, decreased responses to bacillus Calmette-Guérin (BCG) have been shown in children sensitized *in utero* to *Schistosoma hematobium* (Malhotra et al., 1999). Furthermore, children born to mothers infected with *S. mansoni* presented lower anti-measles antibody levels (Ondigo et al., 2018). Altogether, these data suggest that populations where Schistosomiasis is endemic are vulnerable to vaccine failure, and as a consequence, susceptible to deaths due to preventable infectious diseases. Furthermore, endemic regions may require an adjusted immunization regimen in order to ensure optimal protection against diseases.

Similarly, mouse studies have shown reduced protection from BCG vaccine against *Mycobacterium tuberculosis* challenge in mice infected with *S. mansoni (Elias et al., 2005),* while infection during gestation in offspring has shown increased susceptibility to subsequent *S. mansoni* infection (Attallah, Abbas, Dessouky, El-emshaty, & Elsheikha, 2006). Many authors have described a state of hyporesponsiveness to homologous antigens that has been attributed, in part, to immune factors acquired from the mother and previous antigen contact (Santos et al., 2010). Previous sensitization of murine offspring to antigens from mothers infected with other parasitic infections such as *Wuchereria bancrofti* and/or *Plasmodium falciparum* have shown a Th-2 biased (higher production of *il-4* mRNA and IgE antibody) response to diverse antigens when compared to mice from uninfected mothers (Desowitz, Elm, & Alpers, 1993). However, the effects of schistosomiasis during pregnancy in the immune response in offspring and to heterologous antigens remain poorly defined (Friedman, Mital, Kanzaria, Olds, & Kurtis, 2007). *S. mansoni* infection elicits host responses that are similar in human and mice, so the murine model of schistosomiasis is useful to determine the mechanisms by which prenatal infections induce diminished postnatal immune responses. Moreover, the use of mouse as model is based on the comparable stages of gestation which includes implantation, placentation and parturition and the similarity that exists between rodent and human immune cells in the fetal and placental interface and the pathways regulating the fetus development (Krishnan, Nguyen, & McComb, 2013).

In this study we aim to define the effects of perinatal *S. mansoni* infection in offspring during homeostasis and determine whether maternal infection causes a deleterious or beneficial effect in the response to immunization with heterologous antigens. We have established a novel experimental murine model of maternal schistosomiasis in IL-4 dual reporter mice. Our data from this model demonstrate that maternal schistosomiasis reduces primary and memory cellular

responses to immunization with commercially available tetanus/diphtheria vaccine at diverse timepoints of the rodent's lifetime.

Results

Maternal *Schistosoma mansoni* infection results in *in utero* egg antigen sensitization and reduced plasma cell production in offspring

We infected 4get homozygous (IL-4 reporter mice on a BALB/c background) female mice with a low dose of *S. mansoni* (35 cercariae) as described in Materials and Methods. At four weeks post infection (the timepoint where egg production begins) they were paired with a naïve KN2 homozygous (IL-4 deficient) male. At 6 weeks post infection females were tested for antibodies specific to Schistosoma egg antigen (SEA) to confirm infection. Mixed gender and age matched pups from infected and control uninfected mothers were weaned at 28 days to maximize survival and lactation period. At 28-35 days old pups were sacrificed for steady state studies or immunized with adjuvanted commercial vaccine Tetanus/diphtheria. Egg antigen (SEA) specific antibodies were tested at 35, 90 and 180 days in pups from infected and uninfected mothers (Figure 3.1). As expected, pups from mocked infected (controls) showed no antigen specific response to SEA at any of the timepoints. In contrast, pups from infected mothers exhibited anti- SEA IgG1 antibody at 35, 90 and 180 days of age. In some cases, at 180 days of age, some of the pups exhibited no detectable anti-SEA IgG1 (Figure 3.1 A), indicating heterogeneity in the humoral response to egg antigens. Next, we wondered if the antigen specific antibody was maternal derived or self-derived. For this, we mated 4getKN2 (infected or uninfected) females and KN2 homozygous males, for a chance of 50% to obtain KN2 homozygous or 4getKN2. Since KN2 homozygous are deficient of IL-4, they should have class switching deficiencies. We observed that at 35, 60 and 90 days KN2 homozygous and 4getKN2 had detectable anti SEA IgG1 antibodies (Figure 3.1 B). Interestingly,

we also observed that at steady state, pups from infected mothers had significantly ($p=0.045$) increased number of cell secreting antibody against SEA (Figure 3.1 C).

Maternal *S. mansoni* infection leads to diminished bulk plasma cells and follicular dendritic cells at steady state and correlates with reduced steady state plasma cells

In order to determine the consequences of maternal schistosomiasis in offspring during homeostasis, we analyzed the cellular immune responses in various organs (popliteal lymph node, hepatic lymph node, spleen and peripheral blood mononuclear cells). We observed that sensitization *in utero* to *S. mansoni* egg antigens correlated to a diminished bulk plasma cell population at steady state in both popliteal and hepatic lymph node (Figure 3.2), with a reduction of 42% of plasma cells in popliteal lymph node and 60% reduction of total plasma cell in hepatic lymph node. Plasma cells require cell extrinsic factors from epithelial cells such as B-cell activating factor (Baff) (Xu et al., 2007). Baff is secreted by cells such as follicular dendritic cells. In addition, follicular dendritic cells trap immunocomplexes via Fc and Complement receptors (Tew, Wu, Fakher, Szakal, & Qin, 2001). Previous observations have established that follicular dendritic cells (FDC) serve as sites of B cell antigen capture (Suzuki, Grigorova, Phan, Kelly, & Cyster, 2009), and that persisting antigens trapped by FDC induce somatic hypermutation (Wu et al., 2008). Since we found that pups born to infected mothers have reduced plasma cells in lymph nodes, we sought to determine whether this is accompanied by modification in the stromal cell population of follicular dendritic cells (defined as CD21/35, complement receptor 2 and 1, respectively and FDC-M2) via tile confocal microscopy. Indeed, in pups from infected mothers a marked decrease of intensity in the markers for FDC is observed (Figure 3.2 and 3.3). In addition to reduced intensity of CD21/35 and FDC-M2 in naïve peripheral lymph nodes, we also observed reduced area (Figure 3.2). To confirm the confocal tile scan results, we analyzed lymph nodes by

flow cytometry and confirmed that offspring born to *S. mansoni* infected mothers have reduced frequency and cell number of FDC in peripheral lymph nodes.

Invariant NK T cells are the source of IL-4 in the peripheral lymph node at steady state

We observed reduced Th2 cells at steady state in the lymph node and PBMC of infected pups in comparison to the pups from infected mothers. We analyzed a diverse pool of cells including gamma delta T cells, alpha beta T cells, NK, NK T cells and iNKT cells as possible source of IL-4 in steady state. We observed very little production and secretion of IL-4 in all cells except for the invariant NK T cells population (Figure 3.4). Moreover, there was a marked decrease in both production and secretion of IL-4 in the lymph node in offspring from infected mothers, with a 5 fold reduction in the secretion ability of iNKT cells from pups that were antenatally exposed to SEA antigens.

Offspring born to infected mothers have reduced Th2 development following primary immunization with Tetanus/diphtheria

Having confirmed that maternal infection induces changes in the immune response in offspring at steady state, we wondered whether the immune response to heterologous antigens could be affected by *S. mansoni* maternal infection. We immunized 28 to 35 day old 4get/KN2 pups subcutaneously (rear footpad injection) from infected and uninfected mothers with $1/10^{th}$ of the human dose of adjuvanted Tetanus/Diphtheria vaccine. At eight days post-immunization we observed that pups from infected mothers exhibited reduced germinal centers in popliteal lymph nodes as well as reduced IL-4 production in the draining lymph node (Figure 3.5). Moreover, offspring born to infected mothers presented significantly reduced IL-4 transcription (Figure 3.5). As suggested by the confocal tile scans, mice *from S. mansoni* infected mothers had significantly

reduced germinal center formation by flow cytometry (Figure 3.7). In addition to reduced frequency, immunofluorescence analysis of popliteal lymph nodes revealed that germinal center size was reduced in the draining lymph node of mice born to infected mothers. Subsequently, we analyzed the T follicular cells (Tfh) and observed a significant reduction of Tfh cells at 8 days post-immunization in offspring from infected mothers (Figure 3.6). Additionally, we observed a defect in the memory B cell response (IgG1+ B cells) in the pups from infected mother (Figure 3.7), and also a significant defect in the bulk memory B cell population (CD27+ CD19+ cells) (Figure 3.7 B and C). This suggests that maternal infection correlates with an adverse response to heterologous antigens such as diphtheria antigens and tetanus antigens following a primary immunization.

S. mansoni **infection during pregnancy correlates with a weakened offspring's immune response 14 days post-immunization**

In order to determine the effects of infection at the peak of the antibody response, we immunized 4get/KN2 pups with adjuvanted Tetanus/Diphtheria. 14 days post-primary immunization we observed a defect in Tfh cell population (Figure 3.8 A), with a reduced Th2 response (Figure 3.8 B) and decreased plasma cells in pups born to infected mothers (Figure 3.8 C). Maternal infection correlates with decreased production of the cytokine IL-4 visualized by tile confocal imaging (Figure 3.9). Moreover, these defects correlated with a decreased frequency of FDC, confirmed by flow cytometry and immunofluorescence (Figure 3.8 D). In addition to reduced frequency, pups from infected mothers exhibited reduced area of FDC in draining peripheral lymph nodes.

Examination of memory B cells in different tissues revealed that the memory B cell population is significantly decreased in offspring from infected mothers. This reduction could be detrimental to mount an effective secondary response.

Schistosomiasis during pregnancy alters the memory response to heterologous antigens in the offspring

Immunological memory is key to a protective immune response. Our previous work has demonstrated that subcutaneous immunization induces a persistent germinal center that continues to generate antigen specific B cells at a low level, and that these cells are critical for an accelerated secondary immune response (Fairfax et al., 2015). Examination of the response to T/D during maternal schistosomiasis revealed that at 60-90 days post immunization, mice born to infected mothers exhibited a defect in germinal center persistence (Figure 3.10) accompanied by reduced Tfh cell frequencies (Figure 3.10). Altered Th2 responses (IL-4 transcription) was also significantly reduced in pups from infected mothers compared to their uninfected counterparts. This correlated with a marked defect in CD21/35 expression in stromal (mesenchymal) cells (Figure 3.11) and overall reduced frequency of FDC in the draining lymph node (Figure 3.11).

Secondary immune responses to Tetanus/Diphtheria vaccines are compromised in pups born to S. mansoni infected dams

In order to analyze the secondary response in maternal infection, pups were immunized for the first time 28 to 35 days old. After over 60 days, pups were challenged with a second dose of adjuvanted Tetanus/Diphtheria vaccine subcutaneously (Figure 3.12). Indeed, in comparison to pups born to uninfected (controls) mothers, pups from infected dams had reduced germinal center (Figure 3.12 B). Moreover, FDC were reduced approximately 40% in pups from infected mothers. Consistent with previous timepoints, fluorescence intensity of complement receptor 1 and 2 (CD35

and CD21) were reduced in draining peripheral lymph nodes. This was accompanied by decreased fluorescent intensity of FDC-M2 (Figure 3.12). Interestingly, the reduction in cellular T and B during a recall response correlated to significant reduction bulk memory B cells (Figure 3.13 A) and IgG1 memory B cells (Figure 3.13 B) in spleen, which highlights the relevance of the memory pool for cellular responses during a secondary response.

Discussion

In this study we report the generation of a maternal *Schistosoma mansoni* infection model in dual IL-4 reporter mice. Our results indicate that *in utero* egg antigen sensitization occurs, which is consistent to what has been published by other authors (Santos et al., 2016) and confers a state of immune-hyporesponsiveness in the offspring at steady state, this study does not differentiate between antigen exposure and sensitization occurred through breastfeeding or during gestation; however, it has been previously suggested that exposure to parasitic antigens either *in utero* or via breast milk diminishes the heterologous response (Santos et al., 2016). We observed anti- *S. mansoni* egg antigen specific IgG1 (the dominant isotype produced against egg antigens (Fairfax et al., 2015)) at 35, 90 and 120 days of age in mice from infected mothers and no detectable titers in mice from uninfected female controls, suggesting passive transfer of maternal antibody. Moreover, our findings that infected pups have SEA specific antibody secreting cells suggest the possibility of maternal transfer of cells or the persistence of antigens in the offspring and the offspring's intrinsic capacity to produce antigen specific humoral responses. Future work will determine what proportion of these cells are of maternal versus fetal origin. The placental transfer of different IgG subclasses has been well documented (Lostal Gracia, Larrad Mur, & Perez Gonzalez, 1993; Okoko et al., 2001; Pitcher-Wilmott, Hindocha, & Wood, 1980), and IgG1 antibody elimination half-life is estimated to be 18-21 days (Ryman & Meibohm, 2017).

Nevertheless, in our model, we observed maternal antibodies in the KN2 homozygous (IL-4 deficient) pups at 35 and 60 days old, suggesting a sustained antibody response to SEA from either maternal antibodies or cells. The presence of an antigen specific humoral response can potentially translate to protection, or alternatively, inhibit vaccination induced protection, as is the case for measles vaccination, in humans and other mammals (Niewiesk, 2014).

Previous reports have documented the presence of profibrotic biomarkers in cord blood from neonates born to mothers infected with *S. japonicum*, which was attributed to the fetal response to schistosome antigens, as the transfer of collagen markers is unlikely (McDonald et al., 2014). Interestingly, our assessment of the immune response of offspring at steady state revealed that bulk antibody responses were impaired in offspring from infected mothers in comparison to their uninfected counterparts. This state correlated to diminished follicular dendritic cell populations in the peripheral lymph nodes. In addition to this, the area of complement receptor 1 and 2 expressed by FDC in B cell follicles is also reduced at steady state. Collectively, these findings are consistent with a role for FDCs in the impaired maintenance of plasma cells seen in offspring from infected mothers. FDC are the stromal cells located in the germinal center that retain antigen for a long period and bind B cells to prevent apoptosis, and have previously been demonstrated to be fundamental for maintenance of plasma cells and long-lived memory B cells (Al-Alwan et al., 2007). Recent work in our laboratory has demonstrated that IL-4 is required for FDC maintenance and positioning at steady via induction of lymphotoxin α/β (Cortes-Selva, Ready, Gibbs, Rajwa, & Fairfax, 2019). In light of that, we sought to determine the cellular source of steady state lymph node IL-4 production in this model. We found that iNKT cells are the major producers of IL-4 in control offspring, and that secretion of IL-4 is almost eliminated in offspring born to infected mothers. This data suggests that the observed defects in FDC numbers and

stimulatory capacity may be due to insufficient IL-4 during lymph node development and maturation. Bolstering this is the finding that there is a significant reduction in IL-4 competent (GFP+) CD4 T cells in the lymph node of these mice. This evidence suggests that prenatal helminth sensitization has a lasting effect on offspring immunity, and that offspring from mothers that were infected during pregnancy (Lacorcia & Prazeres da Costa, 2018) could be at a higher risk of infection with various pathogens due to a defect in homeostatic immunity, which will be the focus of future studies in our lab.

We further evaluated the immune response following primary immunization with a commercially available and alum adjuvanted Tetanus/diphtheria vaccine used in clinical settings. There is extensive evidence in humans of impaired response to immunization (Labeaud, Malhotra, King, King, & King, 2009; Ondigo et al., 2018), but little is known of the mechanism(s) controlling this defect. We observed that post primary immunization, the pups from infected mothers exhibit a defect not only in expansion of FDCs, but also in T follicular helper cells and germinal center formation. We hypothesize that the combined FDC and Tfh deficiency leads to the diminished response to primary immunization, as well as memory T and B cell responses. Indeed, at 8 days post immunization, there is a reduction of bulk memory B cells and $IgG1^+$ memory B cells, which could potentially have an impact in the long-term maintenance of humoral immunity necessary for protection following vaccination.

We also explored the response to immunization at 14 days, which has been determined as the peak of the humoral response for a number of antigens (Fairfax et al., 2015). Consistent with our previous observations, germinal center formation in reactive lymph node was impaired in 4get/KN2 pups from infected mothers in comparison to pups from naïve mothers. Acute antigen exposure causes lymphocyte proliferation, which is followed by a pool of quiescent long-lived IL-

$7R^+$ memory cells capable of potent response to challenge with the same antigen. (Seddon, Tomlinson, & Zamoyska, 2003). We have previously determined that in a type 2 response secondary TFH cells are generated in large part by recruitment of circulating Th2 memory T cells (IL-$7R^+GFP^+$) back to the lymph node, and that their interaction with memory B cells is critical to the secondary plasma cell response (Fairfax et al., 2015). In our model of maternal schistosomiasis, we observed a diminished pool of both populations of IL-$7R^+GFP^+CD4^+$ (memory Th2 cells) and IL-$7R^-GFP^+CD4^+$ cells (Th2 effector cells). This was accompanied by a reduction in the frequencies of Tfh cells and plasma cells, as well as a diminished secretion of IL-4 cytokine in the reactive lymph node. In addition to the defects observed in T cells, we also observed reduced FDC frequencies and a reduced bulk memory B cell population. Overall, this indicates that signals required for mounting a robust adaptive immune response to vaccination were dampened following prenatal exposure to *S. mansoni antigens*. However, the specific molecular mechanism responsible for these defects are still unclear, although our data suggests a role for iNKT cell IL-4 production in FDC maintenance the data is not definitive. Future work will focus on identifying the specific homeostatic role of iNKT cell IL-4, and the molecular mechanism that underlie bot iNKT cell production of IL-4, and diminished FDC cell function in the context of maternal infection. There is a body of literature supporting the many effects of the prenatal environment on inflammatory diseases during adolescence and adulthood, it has been hypothesized that inflammatory responses and infections during pregnancy might alter epigenetic profiles in the fetus (Claycombe, Brissette, & Ghribi, 2015). Future studies linking the epigenetics effects of schistosomiasis infection during pregnancy and immune response in offspring will help elucidate potential pathways involved in the immune hypo-responsiveness observed in this model.

One of the key goals of vaccination is to induce an immunological memory that is protective upon re-challenge with the same antigen (Bevan, 2011; Castellino, Galli, Del Giudice, & Rappuoli, 2009; Sallusto, Lanzavecchia, Araki, & Ahmed, 2010). Hence, we explored whether there was a difference between pups from infected and control mothers in the development of a sustained germinal center and maintenance of memory B and T cell pools. Pups were immunized and their immunological response was assessed at >60 days post immunization. Surprisingly, offspring from infected mother mounted a weaker cellular response, with significantly smaller germinal centers that correlated to significantly reduced long lived Tfh, memory Th2 cells, and FDCs as well as reduced complement receptor 2/1 (CD21/35) expression. Although there was a trend for reduction of precursor bulk memory T cells ($CXCR5^{+}PD\text{-}1^{-}$) these were not significantly altered among pups from infected mothers in comparison to pups from uninfected mothers.

Finally, we assessed the secondary response to tetanus/diphtheria and consistently observed a deficit in the expansion of the secondary Tfh population and FDCs following secondary challenge. Antigen persistence is known to be critical for maintenance of memory B cells and antigens persist on FDC as ag-ab complex that are available for ongoing stimulation of B cells, so the consistent deficiency in FDC populations is of critical importance. In here, we have established a rodent model of schistosomiasis maternal infection, future studies will focus on the functional capacities of follicular dendritic cells and T cells to understand the immune deficiencies during primary and secondary immunizations during maternal sensitization with helminths.

Materials and Methods

Mice strains and *in vivo* treatments

4get homozygous ($Il4^{tm1Lky}$, The Jackson Laboratory), KN2 homozygous ($Il4^{tm1(CD2)Mmrs}$, gift from Markus Morhs (Mohrs, Wakil, Killeen, Locksley, & Mohrs, 2005)) and 4get/KN2 were

bred in-house under specific pathogen free (SPF) conditions at Purdue University. Experimental procedures involving mice were approved by the Purdue Animal Care and Use Committee (PACUC). Homozygous 4get females were either infected with a low dose (35 cercariae) of *Schistosoma mansoni* or mock infected. Infection was performed by exposing mice percutaneously to the parasite cercariae for 25 minutes. Infected and uninfected females (as controls) were bred with naive KN2 homozygous male mice four weeks post-infection and resulting 4get/KN2 pups were weaned at 28 days old. A mix of age-matched female and male pups either from control or infected females were sacrificed at 29-35 days old for steady state studies. Other experimental mice were immunized with $1/10^{th}$ of the human dose of Tetanus/Diphtheria commercial vaccine (Tetanus and Diphtheria Toxoids Adsorbed, MassBiologics) sub cutaneous (s.c.) in the rear footpad and mice were sacrificed at 8, 14, and over sixty (memory) days post-immunization. Secondary immunizations were performed at over 60 days post primary immunization and mice were sacrificed 3 days post-secondary immunization.

Isolation of cells and flow cytometry

Cells were isolated from popliteal lymph node (pLN), hepatic lymph node (hLN), whole blood and spleen. For follicular dendritic cells (FDC) analysis tissues were digested as previously described ((Cortes-Selva, Ready, Gibbs, Rajwa, & Fairfax, 2018; Hara et al., 2012). Briefly, lymph nodes were digested at 37°C for 20 minutes, with occasional inversion of the contents and filtered through 100 μm filters. Single cell suspensions were stained with surface markers and intracellular staining was performed as previously described (Glatman Zaretsky et al., 2009). Antibodies conjugated with the following fluorochromes were used: allophycocyanin, allophycocyanin-Cy7, Pacific blue, Brilliant Violet, FITC, Phycoerythrin, PE/Cy7, PE/Cy5. The following antibodies from eBioscience, BD Biosciences, Biolegend were used: CD4 (RM4-5), CD19 (1D3), CD138,

(281-2), IgG1 (A85-1), IgD (11-26), CD11c (N418), F4/80 (BM8), GL7 (GL7), CD31 (390), podoplanin (eBIO 8.1.1), FDC-M2 (FDC-M2), CD21/35 (7G6), PD-1 (J43), CXCR5 (2G8), PNAd (MECA-79) and IL-21R/FC chimera (R&D Systems; 596-MR-100).

Immunofluorescence microscopy

Whole tissue (popliteal lymph node or hepatic lymph node) was collected and placed in Tissue-Tek optimum cutting temperature compound (OCT) (Thermo Scientific) and frozen in liquid nitrogen. Serial cryostat sections (10μm) were collected using Leica CM 1850. Sections were then air-dried and fixed in ice-cold 75% acetone/25% ethanol for 5 mins. Sections were rehydrated in PBS for 5 to 10 minutes and blocked using biotin blocking kit (Vector Laboratories) followed by incubation with 1%(v/v) in PBS of rat and rabbit serum. Staining with appropriate antibodies was done overnight at 4°C followed by secondary staining for 1 hour at room temperature. Sections mounting was done using ProLong anti-fade reagents (Life Technologies). Images were acquired with a Leica TCS Sp5 Laser Scanning Microscopy with an average grid size of 3x3. Images were taken with a 20x objective at a resolution of 1024x1024. Image post-processing was done using Fiji is Just ImageJ software (1.47v).

ELISA

Schistosoma mansoni egg antigens (SEA), Tetanus and diphtheria-specific IgG1 endpoint titers were determined by enzyme-Linked immunosorbent assay (ELISA) using the mAb X56 (BD) and Immulon 4HB plates (Thermo Fisher Scientific) as previously described (Cortes-Selva et al., 2018). Briefly, plates were coated with 2μg/mL tetanus (List Labs), diphtheria (Sigma) and SEA. The following morning plates were blocked with 1% milk and incubated with serial dilutions of primary antibody, followed by incubation with anti-mouse IgG1 ads-HRP antibody (Southern

Biotech) and ABTS substrate. Plates were read at 405 nm at room temperature on a BioTek plate reader.

ELISPOT

For ELISPOTS, single cell suspension from spleen were cultured at different dilutions in complete media for 24 hours in MultiScreen-HA plates (Millipore, Bellerica, MA). The plates were previously coated with 2 µg/ml of Schistosoma egg antigen. Following 24 hours incubation with splenocytes, plates were washed and anti mouse IgG1 HRP antibody was applied for 2 hours at 37 degrees. Bound antibody was detected with AEC Chromogen (Sigma) per manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using either a non-parametric Mann-Whitney test, unpaired Student's t test or ANOVA, Bonferroni's multiple comparison test based on the distribution of the data. p values ≤ 0.05 were considered statistically significant. Graph generation and statistical analyses were performed using Prism (GraphPad v5.0).

References

Al-Alwan, M., Du, Q., Hou, S., Nashed, B., Fan, Y., Yang, X., & Marshall, A. J. (2007). Follicular dendritic cell secreted protein (FDC-SP) regulates germinal center and antibody responses. J Immunol, 178(12), 7859-7867.

Attallah, A. M., Abbas, A. T., Dessouky, M. I., El-emshaty, H. M., & Elsheikha, H. M. (2006). Susceptibility of neonate mice born to Schistosoma mansoni-infected and noninfected mothers to subsequent S. mansoni infection. Parasitol Res, 99(2), 137-145. doi:10.1007/s00436-006-0127-x

Bevan, M. J. (2011). Understand memory, design better vaccines. Nat Immunol, 12(6), 463-465. doi:10.1038/ni.2041

Castellino, F., Galli, G., Del Giudice, G., & Rappuoli, R. (2009). Generating memory with vaccination. Eur J Immunol, 39(8), 2100-2105. doi:10.1002/eji.200939550

Claycombe, K. J., Brissette, C. A., & Ghribi, O. (2015). Epigenetics of inflammation, maternal infection, and nutrition. J Nutr, 145(5), 1109S-1115S. doi:10.3945/jn.114.194639

Colley, D. G., Bustinduy, A. L., Secor, W. E., & King, C. H. (2014). Human schistosomiasis. Lancet, 383(9936), 2253-2264. doi:10.1016/S0140-6736(13)61949-2

Cortes-Selva, D., Ready, A., Gibbs, L., Rajwa, B., & Fairfax, K. C. (2018). IL-4 Promotes stromal cell expansion and Is critical for development Of A Type-2, but not a type 1 immune response. Eur J Immunol. doi:10.1002/eji.201847789

Desowitz, R. S., Elm, J., & Alpers, M. P. (1993). Plasmodium falciparum-specific immunoglobulin G (IgG), IgM, and IgE antibodies in paired maternal-cord sera from east Sepik Province, Papua New Guinea. Infect Immun, 61(3), 988-993.

Elias, D., Akuffo, H., Pawlowski, A., Haile, M., Schon, T., & Britton, S. (2005). Schistosoma mansoni infection reduces the protective efficacy of BCG vaccination against virulent Mycobacterium tuberculosis. Vaccine, 23(11), 1326-1334. doi:10.1016/j.vaccine.2004.09.038

Fairfax, K. C., Everts, B., Amiel, E., Smith, A. M., Schramm, G., Haas, H., . . . Pearce, E. J. (2015). IL-4-secreting secondary T follicular helper (Tfh) cells arise from memory T cells, not persisting Tfh cells, through a B cell-dependent mechanism. J Immunol, 194(7), 2999-3010. doi:10.4049/jimmunol.1401225

Friedman, J. F., Mital, P., Kanzaria, H. K., Olds, G. R., & Kurtis, J. D. (2007). Schistosomiasis and pregnancy. Trends Parasitol, 23(4), 159-164. doi:10.1016/j.pt.2007.02.006

Glatman Zaretsky, A., Taylor, J. J., King, I. L., Marshall, F. A., Mohrs, M., & Pearce, E. J. (2009). T follicular helper cells differentiate from Th2 cells in response to helminth antigens. J Exp Med, 206(5), 991-999. doi:10.1084/jem.20090303

Hara, T., Shitara, S., Imai, K., Miyachi, H., Kitano, S., Yao, H., . . . Ikuta, K. (2012). Identification of IL-7-producing cells in primary and secondary lymphoid organs using IL-7-GFP knock-in mice. J Immunol, 189(4), 1577-1584. doi:10.4049/jimmunol.1200586

Krishnan, L., Nguyen, T., & McComb, S. (2013). From mice to women: the conundrum of immunity to infection during pregnancy. J Reprod Immunol, 97(1), 62-73. doi:10.1016/j.jri.2012.10.015

Kurtis, J. D., Higashi, A., Wu, H. W., Gundogan, F., McDonald, E. A., Sharma, S., . . . Friedman, J. F. (2011). Maternal Schistosomiasis japonica is associated with maternal, placental, and fetal inflammation. Infect Immun, 79(3), 1254-1261. doi:10.1128/IAI.01072-10

Labeaud, A. D., Malhotra, I., King, M. J., King, C. L., & King, C. H. (2009). Do antenatal parasite infections devalue childhood vaccination? PLoS Negl Trop Dis, 3(5), e442. doi:10.1371/journal.pntd.0000442

Lacorcia, M., & Prazeres da Costa, C. U. (2018). Maternal Schistosomiasis: Immunomodulatory Effects With Lasting Impact on Allergy and Vaccine Responses. Front Immunol, 9, 2960. doi:10.3389/fimmu.2018.02960

Lostal Gracia, M. I., Larrad Mur, L., & Perez Gonzalez, J. M. (1993). [IgG subclasses: placental transfer in the full-term neonate and their evolution during the first 3 months of life]. An Esp Pediatr, 38(6), 503-508.

Malhotra, I., Mungai, P., Wamachi, A., Kioko, J., Ouma, J. H., Kazura, J. W., & King, C. L. (1999). Helminth- and Bacillus Calmette-Guerin-induced immunity in children sensitized in utero to filariasis and schistosomiasis. J Immunol, 162(11), 6843-6848.

McDonald, E. A., Cheng, L., Jarilla, B., Sagliba, M. J., Gonzal, A., Amoylen, A. J., . . . Kurtis, J. D. (2014). Maternal infection with Schistosoma japonicum induces a profibrotic response in neonates. Infect Immun, 82(1), 350-355. doi:10.1128/IAI.01060-13

McDonald, E. A., Pond-Tor, S., Jarilla, B., Sagliba, M. J., Gonzal, A., Amoylen, A. J., . . . Friedman, J. F. (2014). Schistosomiasis japonica during pregnancy is associated with elevated endotoxin levels in maternal and placental compartments. J Infect Dis, 209(3), 468-472. doi:10.1093/infdis/jit446

Mohrs, K., Wakil, A. E., Killeen, N., Locksley, R. M., & Mohrs, M. (2005). A two-step process for cytokine production revealed by IL-4 dual-reporter mice. Immunity, 23(4), 419-429. doi:10.1016/j.immuni.2005.09.006

Niewiesk, S. (2014). Maternal antibodies: clinical significance, mechanism of interference with immune responses, and possible vaccination strategies. Front Immunol, 5, 446. doi:10.3389/fimmu.2014.00446

Okoko, B. J., Wesumperuma, L. H., Ota, M. O., Pinder, M., Banya, W., Gomez, S. F., . . . Hart, A. C. (2001). The influence of placental malaria infection and maternal hypergammaglobulinemia on transplacental transfer of antibodies and IgG subclasses in a rural West African population. J Infect Dis, 184(5), 627-632. doi:10.1086/322808

Olveda, D. U., Olveda, R. M., McManus, D. P., Cai, P., Chau, T. N., Lam, A. K., . . . Ross, A. G. (2014). The chronic enteropathogenic disease schistosomiasis. Int J Infect Dis, 28, 193-203. doi:10.1016/j.ijid.2014.07.009

Ondigo, B. N., Muok, E. M. O., Oguso, J. K., Njenga, S. M., Kanyi, H. M., Ndombi, E. M., . . . Colley, D. G. (2018). Impact of Mothers' Schistosomiasis Status During Gestation on Children's IgG Antibody Responses to Routine Vaccines 2 Years Later and Anti-Schistosome and Anti-Malarial Responses by Neonates in Western Kenya. Front Immunol, 9, 1402. doi:10.3389/fimmu.2018.01402

Pitcher-Wilmott, R. W., Hindocha, P., & Wood, C. B. (1980). The placental transfer of IgG subclasses in human pregnancy. Clin Exp Immunol, 41(2), 303-308.

Ryman, J. T., & Meibohm, B. (2017). Pharmacokinetics of Monoclonal Antibodies. CPT Pharmacometrics Syst Pharmacol, 6(9), 576-588. doi:10.1002/psp4.12224

Sallusto, F., Lanzavecchia, A., Araki, K., & Ahmed, R. (2010). From vaccines to memory and back. Immunity, 33(4), 451-463. doi:10.1016/j.immuni.2010.10.008

Santos, P., Lorena, V. M., Fernandes Ede, S., Sales, I. R., Nascimento, W. R., Gomes Yde, M., . . . Souza, V. M. (2016). Gestation and breastfeeding in schistosomotic mothers differently modulate the immune response of adult offspring to postnatal Schistosoma mansoni infection. Mem Inst Oswaldo Cruz, 111(2), 83-92. doi:10.1590/0074-02760150293

Santos, P., Sales, I. R., Schirato, G. V., Costa, V. M., Albuquerque, M. C., Souza, V. M., & Malagueno, E. (2010). Influence of maternal schistosomiasis on the immunity of adult offspring mice. Parasitol Res, 107(1), 95-102. doi:10.1007/s00436-010-1839-5

Seddon, B., Tomlinson, P., & Zamoyska, R. (2003). Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. Nat Immunol, 4(7), 680-686. doi:10.1038/ni946

Siegrist, D., & Siegrist-Obimpeh, P. (1992). Schistosoma haematobium infection in pregnancy. Acta Trop, 50(4), 317-321.

Steinmann, P., Keiser, J., Bos, R., Tanner, M., & Utzinger, J. (2006). Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect Dis, 6(7), 411-425. doi:10.1016/S1473-3099(06)70521-7

Suzuki, K., Grigorova, I., Phan, T. G., Kelly, L. M., & Cyster, J. G. (2009). Visualizing B cell capture of cognate antigen from follicular dendritic cells. J Exp Med, 206(7), 1485-1493. doi:10.1084/jem.20090209

Tew, J. G., Wu, J., Fakher, M., Szakal, A. K., & Qin, D. (2001). Follicular dendritic cells: beyond the necessity of T-cell help. Trends Immunol, 22(7), 361-367.

Tsutsui, T., Yamamoto, T., Hayama, Y., Akiba, Y., Nishiguchi, A., Kobayashi, S., & Yamakawa, M. (2009). Duration of maternally derived antibodies against Akabane virus in calves: survival analysis. J Vet Med Sci, 71(7), 913-918.

Wu, Y., Sukumar, S., El Shikh, M. E., Best, A. M., Szakal, A. K., & Tew, J. G. (2008). Immune complex-bearing follicular dendritic cells deliver a late antigenic signal that promotes somatic hypermutation. J Immunol, 180(1), 281-290.

Xu W, He B, Chiu A, Chadburn, A., Shan, M., Buldys, M., . . . Cerutti, A. (2007) Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. Nat Immunol, 8(3),294-303.

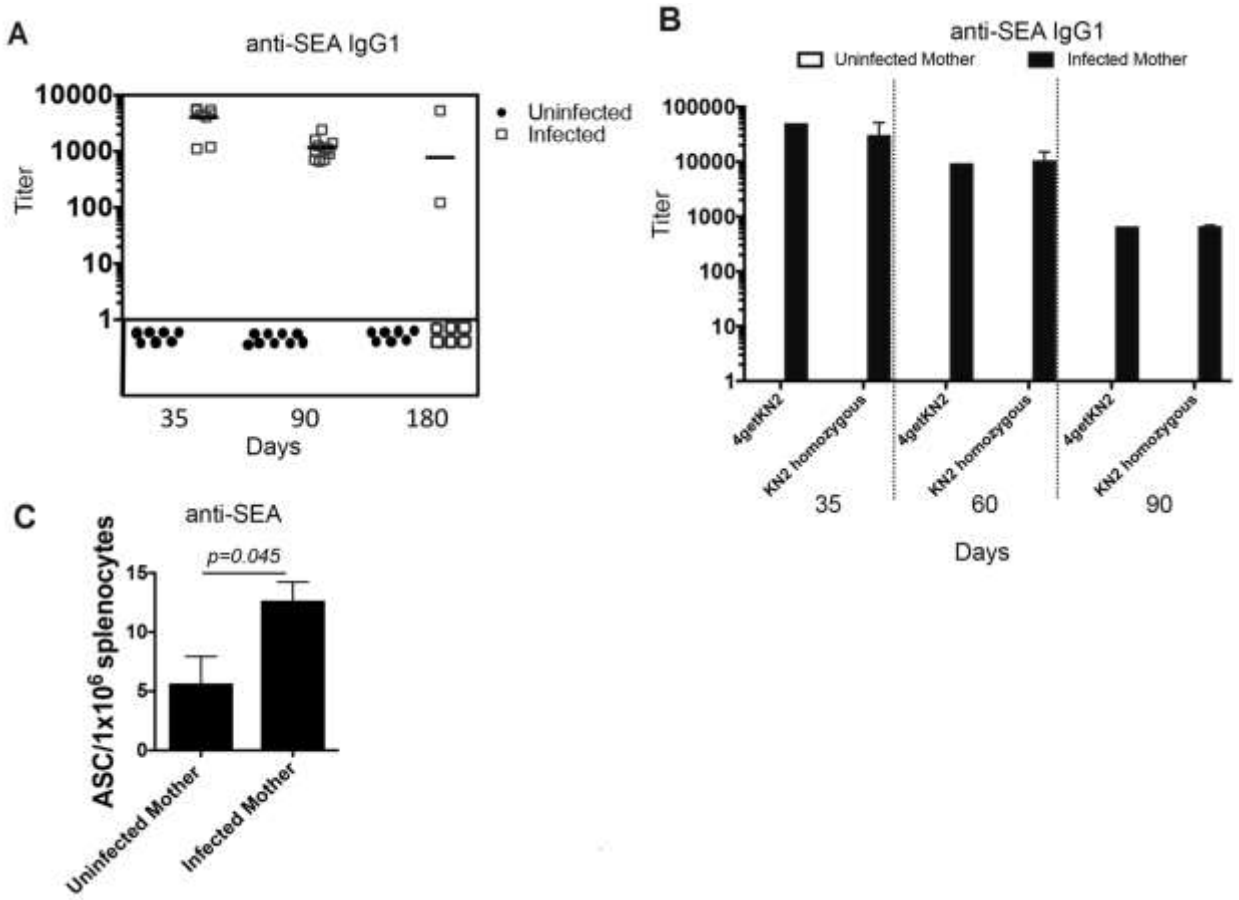


Figure 3.1 Maternal S. mansoni infection leads to a humoral anti-SEA response in offspring.

(A) anti- *Schistosoma mansoni* egg antigen (SEA) specific IgG1 antibody titers in serum of naïve 4get/KN2 mice. Born to infected or uninfected mothers. Each point represents an individual mouse. (B) anti-SEA specific IgG1 antibody titers in naïve KN2 homozygous and 4get/KN2 mice born to infected and uninfected mothers. Error bar denotes mean ± SEM. (C) anti-SEA specific IgG1 secreting cells in splenocytes obtained from naïve 4get/KN2 mice at 28-35 days of age. Significance was determined by Student's t test.

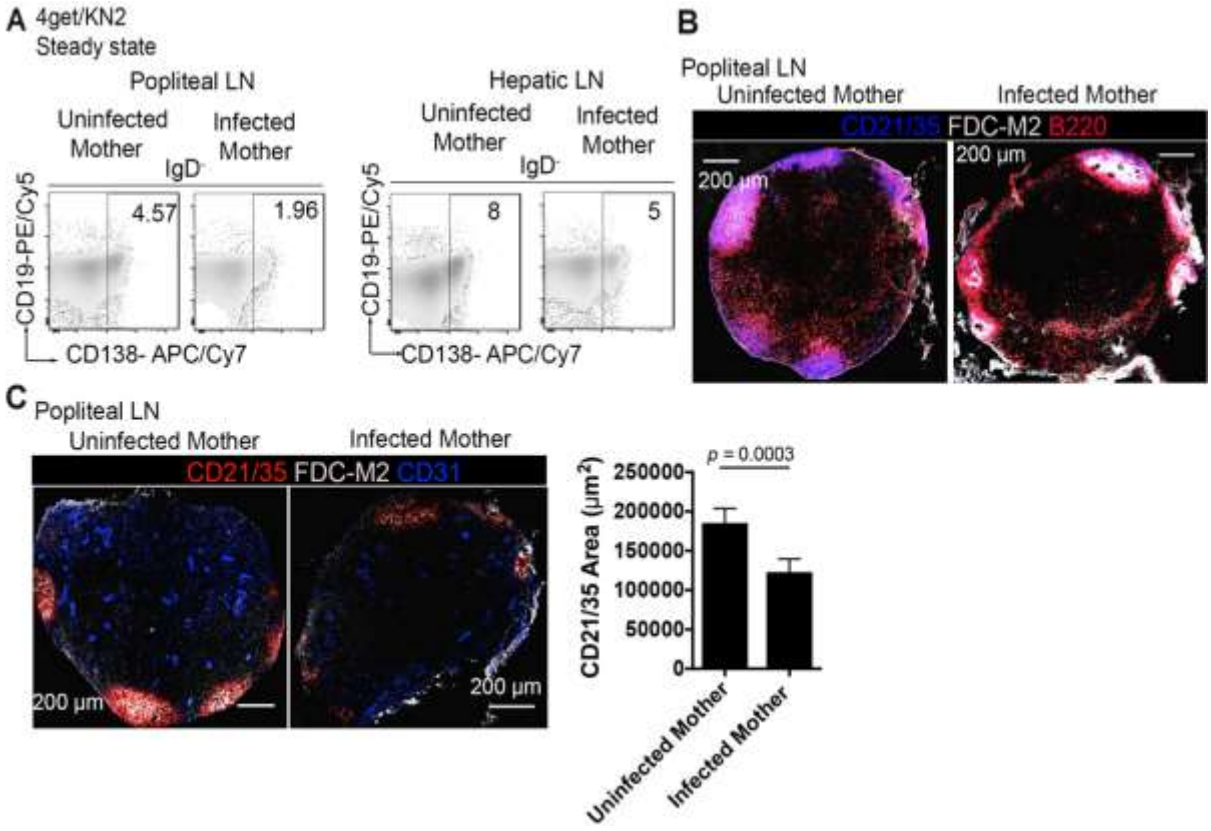


Figure 3.2 Maternal *S. mansoni* infection reduces steady state lymph node plasma and follicular dendritic cells in 28 days old offspring.

(A) Flow cytometry plots of plasma cells (IgD-CD19+/-CD138+) in popliteal and hepatic lymph nodes from naïve 4get/KN2 born to infected and uninfected mothers. (B, C) Tile confocal imaging of naïve popliteal lymph node of 4get/KN2 mice at 28-35 days of age, scale bar: 200 µm. (B) Sections were stained for CD21/35 (blue) FDC-M2 (gray) and B220 (red), (C) Sections were stained for CD21/35 (red) FDC-M2 (gray) and CD31 (blue) with the respective CD21/35 area quantification. (D) Gating strategy for follicular dendritic cells (FDC) and flow cytometry analysis of FDC (CD21/35+FDC-M2+) gated from CD45- with number of FDC in popliteal lymph node from pups od 28-35 days of age.

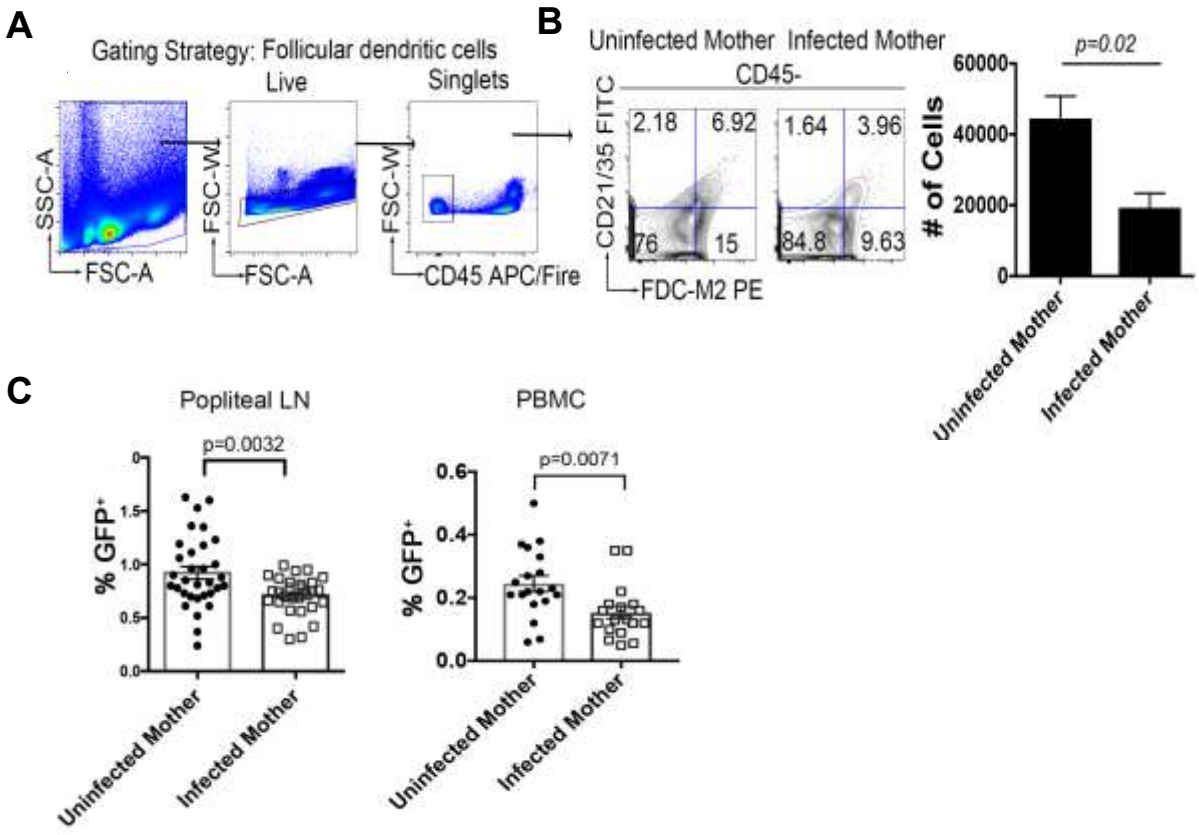


Figure 3.3 Effects of maternal Schistosomiasis in offspring immunity. Naive 4getKN2 pups from infected and uninfected controls were sacrificed at 28-35 days old.

(A) Gating strategy for follicular dendritic cells (FDC) and (B) flow cytometry analysis of FDC (CD21/35+FDC-M2+) gated from CD45- with number of FDC in popliteal lymph node from pups od 28-35 days of age. (C) Frequency of GFP+ in naïve 4get/KN2 mice. Flow plots were concatenated samples, with n >3 mice per group and representative of six independent experiments. Statistical significance was calculated using Student's t test.

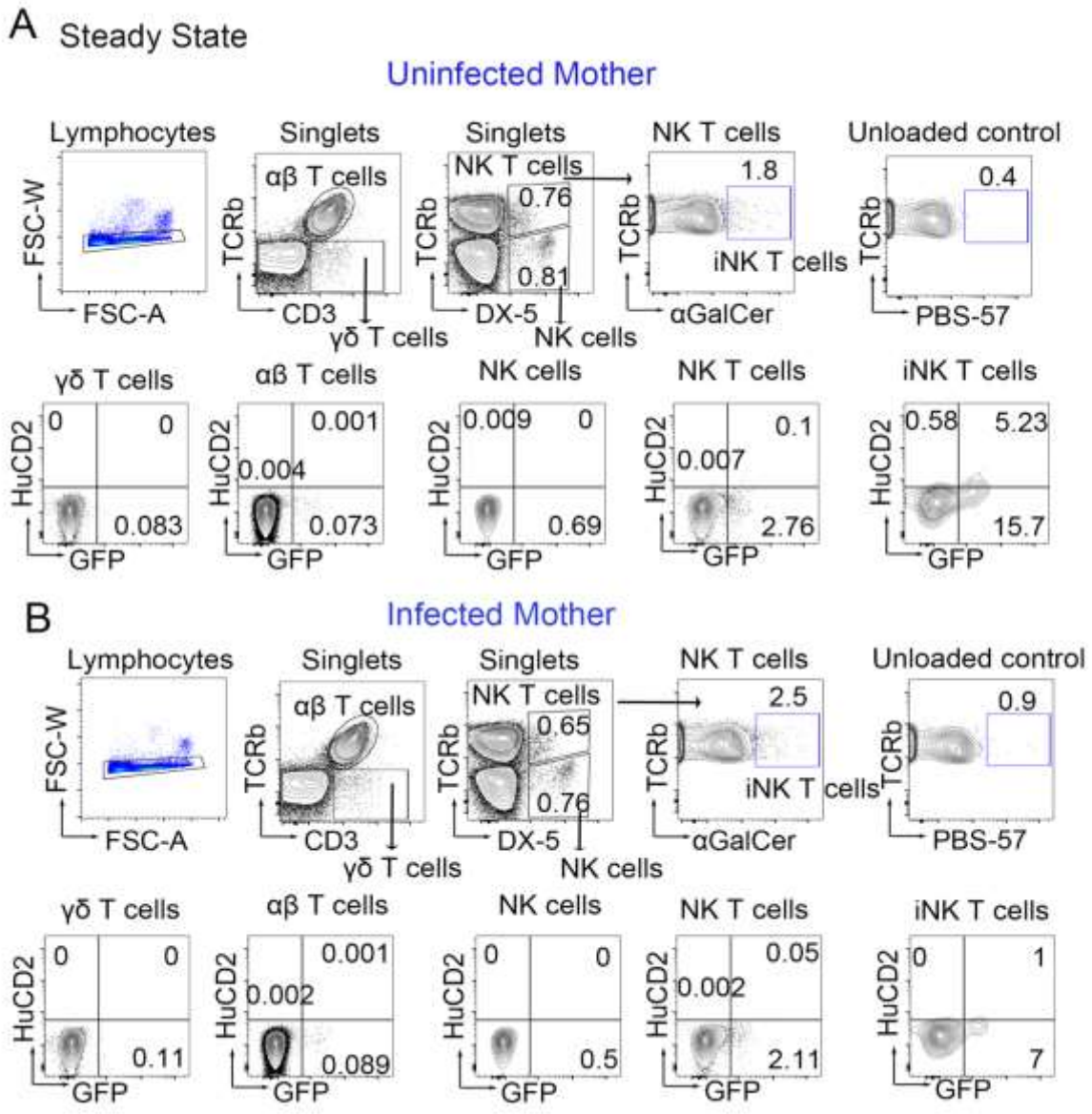


Figure 3.4 iNKT cells are the main cellular source of IL-4 in peripheral lymph nodes at steady state maternal *S. mansoni* infection reduces their secretion of IL-4 at steady state.

Gating strategy in popliteal lymph nodes for invariant iNKT cell (αGalCer^{+}TCRβ^{+} TCRβ^{+}DX-5^{+}), $\alpha\beta$ T cells (TCRβ^{+}CD3^{+}), $\gamma\delta$ T cells (TCRβ^{-}CD3^{+}), NK T cells (TCRβ^{+}DX-5^{+}), NK cells (TCRβ^{-}DX-5^{+}) analyzed by flow cytometry in (A) pups from uninfected mother (B) pups from infected mother at 28-35 days of age. Flow plots are concatenated of n >3 mice from two biologically independent experiments.

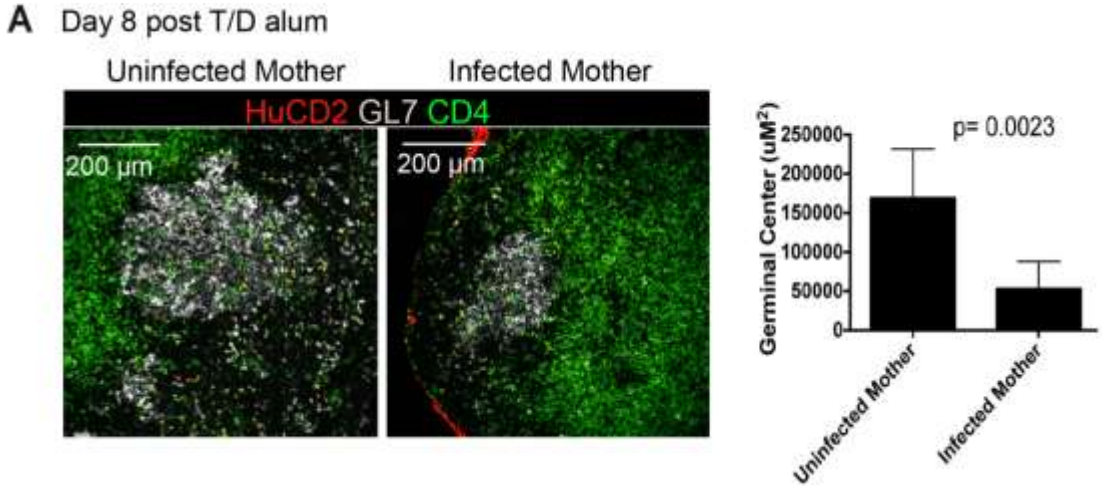


Figure 3.5 Schistosomiasis during pregnancy correlates to diminished Germinal center reactions and IL-4 production in offspring following a primary immunization with alum adjuvanted Tetanus Diphtheria.

Immunological response 8 days post immunization with an alum adjuvanted tetanus diphtheria vaccine in offspring of S. mansoni infected mothers or uninfected control mothers 28-35 days old mice were immunized in the rear footpad with 1/10th of the human dose of an aluminum adjuvanted Tetanus diphtheria vaccine (A) Tile confocal imaging of 10 microns cryosections of popliteal lymph nodes stained for HuCD2 (red), GL-7 (gray) and CD4 (green). Scale bar: 200 µm

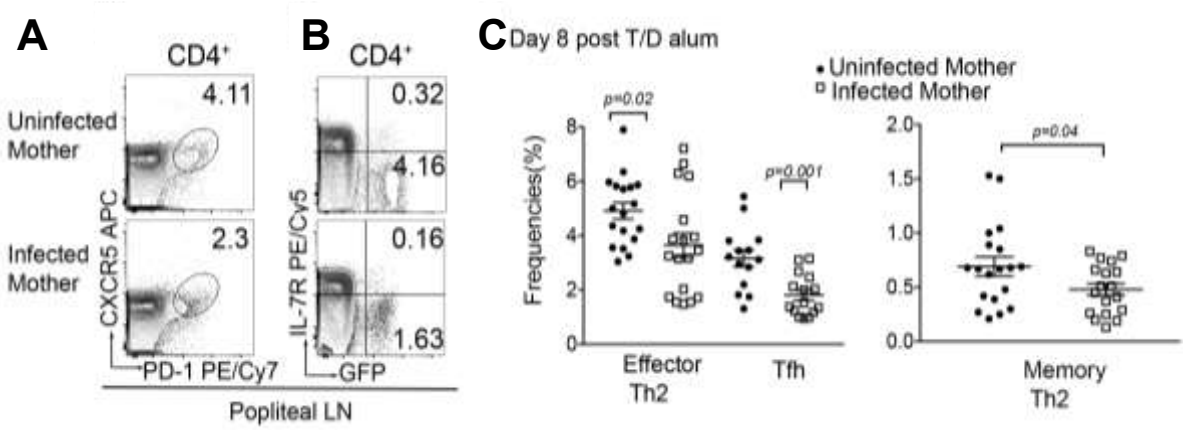


Figure 3.6 Reduced Tfh, Th2 effector and Th2 memory response in offspring from infected mothers.

Flow cytometry analysis from popliteal lymph nodes of (A) T follicular helper cells (PD-1+CXCR5+, gated from CD4+), (B) Th2 effector (GFP+IL-7R-) and Th2 memory (GFP+IL-7R+) (C) Frequencies of effector Th2, memory Th2 and Tfh cells 8 dpi. Flow plots are concatenated from n >3 mice from at least two independent experiments. Statistical significance was calculated using Student's t test.

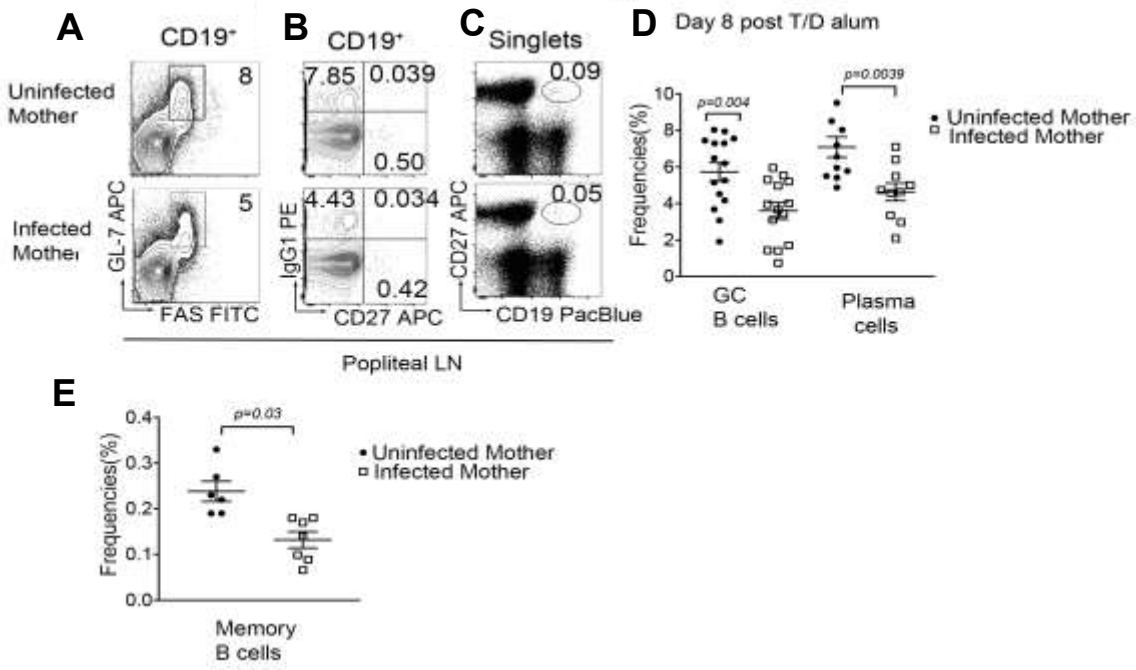


Figure 3.7. Germinal center reactions and memory B cell are reduced in reactive lymph node following antenatal exposure to *S. mansoni* antigens.

(A) Flow cytometry of Germinal center B cells (CD19+GL-7+FAS+) (B) Memory IgG1+CD27+ B cells and Memory IgG2+CD27- B cells (C) Bulk memory B cells (CD27+CD19+) (D) Cell frequencies of GC B cells and plasma cells (E) Cell frequencies of memory Bulk B cells Frequency of bulk memory B cell in popliteal lymph node. Confocal microscopy images are representative of four independent experiments. Flow plots are concatenated from n >3 mice from at least two independent experiments. Statistical significance was calculated using Student's t test.

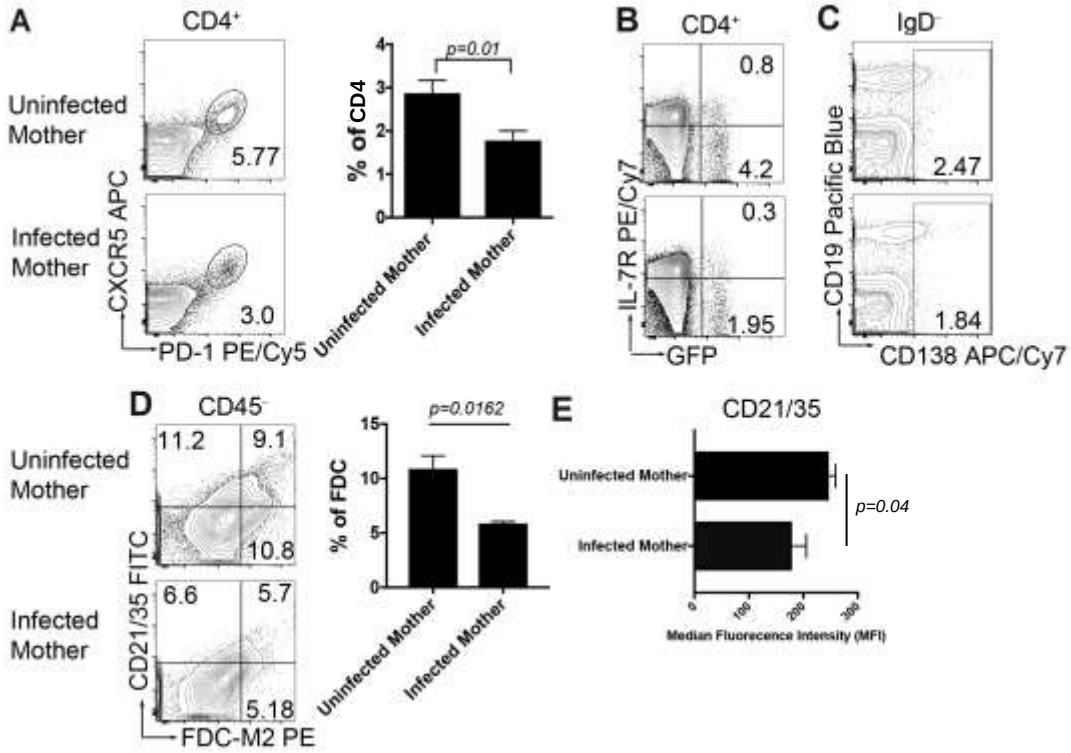


Figure 3.8. Pups from infected mothers exhibit reduced GC B cells, Tfh and FDC 14 days post immunization with Tetanus diphtheria.

(A) Tfh (PD-1^{+}CXCR5^{+}, gated from CD4^{+}) and frequency from draining PLN (B) Th2 effector (GFP^{+}IL-7R^{-}) and Th2 memory (GFP^{+}IL-7R^{+}) from reactive lymph node (C) Plasma cells (IgD^{-} CD19$^{+/-}$CD138^{+}) (D) FDC (CD21/35^{+}FDC-M2^{+}CD45^{-}) frequencies. Flow plots and confocal microscopy data are representative of three independent experiments with n>3 mice per group. Mean bar denotes ± SEM. Statistical analysis was calculated with Student's t test.

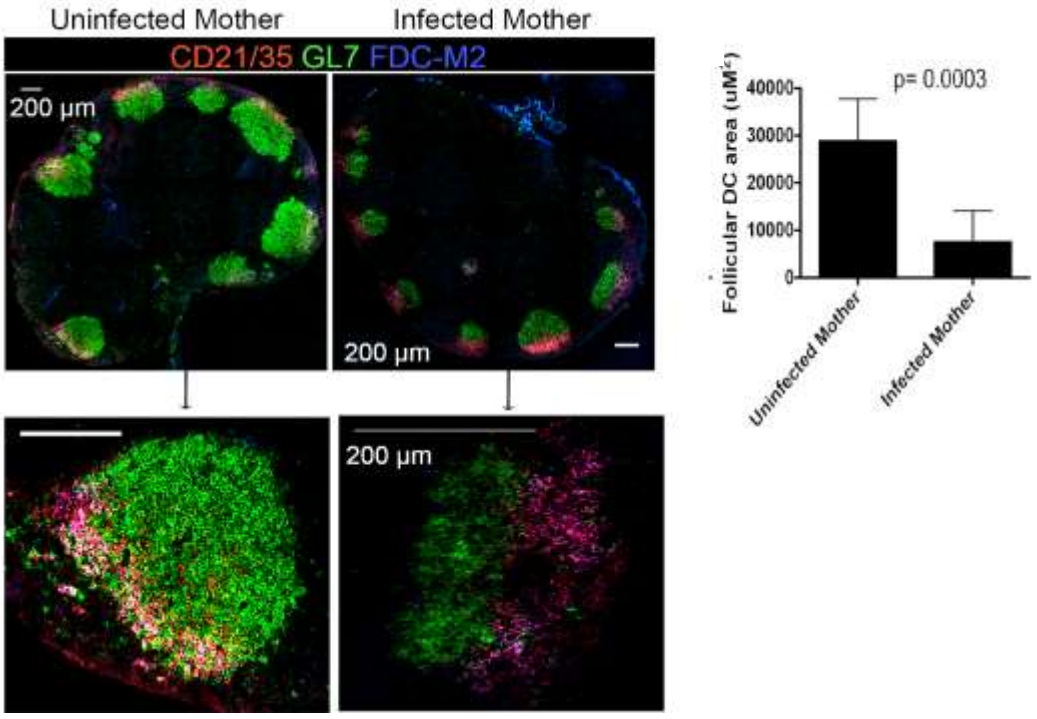


Figure 3.9. Diminished germinal center expansion and follicular cell are characterize the immune response of pups from infected mothers 14 days post immunization).

Cryosections of reactive popliteal lymph node stained with CD21/35 (red), GL-7 (green) and FDC-M2 (blue). Scale bar: 200um.

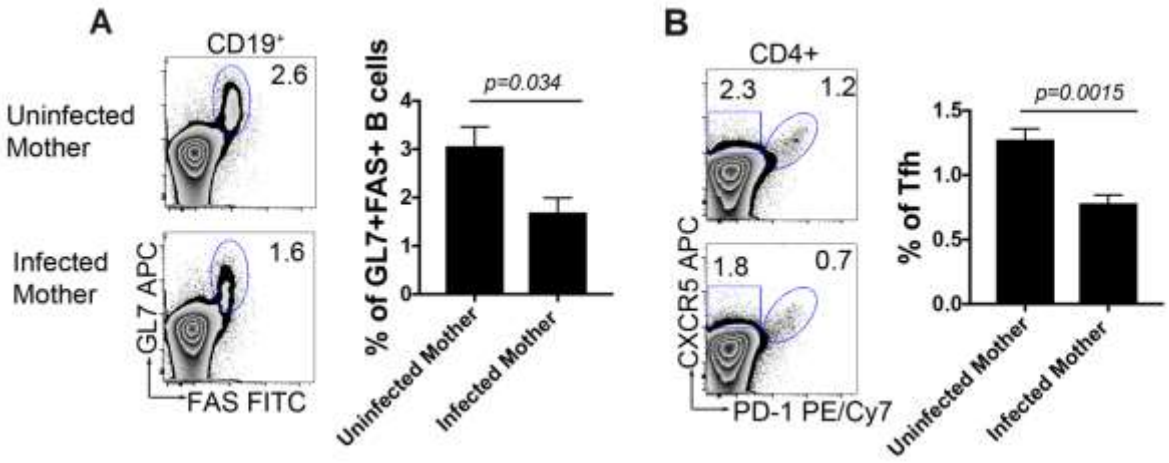


Figure 3.10 Cellular T and B responses are reduced in 4get/KN2 offspring from infected mothers 60 days post primary immunization.

4get/KN2 28-35 days old were immunized in the hind footpad as described in the Materials and Methods, draining popliteal lymph nodes were collected >60 days post primary immunization (A) GC B cells ($CD19^+GL\text{-}7^+FAS^+$) frequencies (B) Tfh ($PD\text{-}1^+CXCR5^+$, gated from $CD4^+$) frequencies. Flow data is concatenated representative of n n>4 mice per group and two independent experiments. Statistical analysis was calculated with Student's t test.

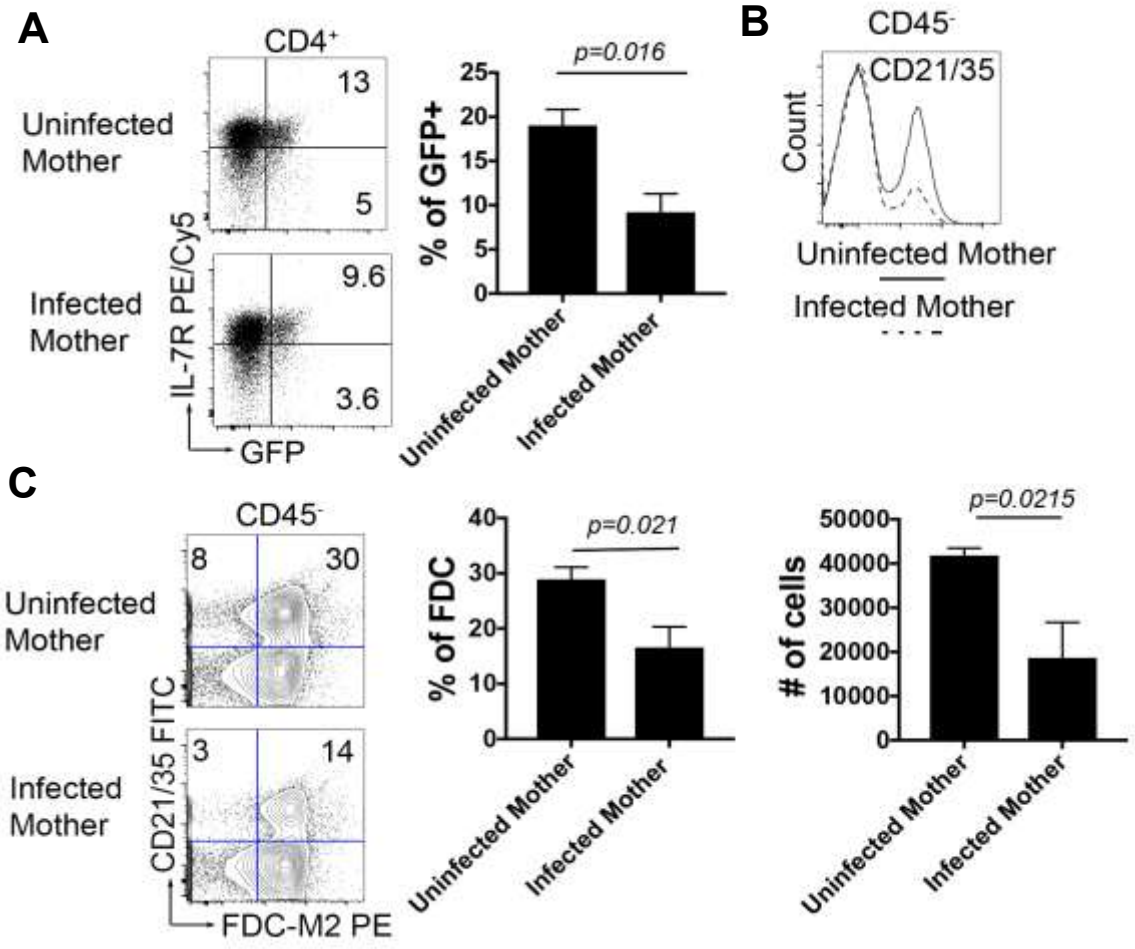


Figure 3.11 Memory T cells, Complement Receptor 1 and 2 and number of Follicular dendritic cells are impaired in a memory response to Tetanus/Diphtheria.

4get/KN2 28-35 days old were immunized in the hind footpad as described in the Materials and Methods, draining popliteal lymph nodes were collected >60 days post primary immunization. (A) Th2 responses ($GFP^{+}IL\text{-}7R^{+/-}$) (B) Flow cytometry analysis of CD21/35 cell count (C) FDC ($CD21/35^{+}FDC\text{-}M2^{+}CD45^{-}$) frequency. Flow data is concatenated representative of n n>4 mice per group and two independent experiments. Statistical analysis was calculated with Student's t test.

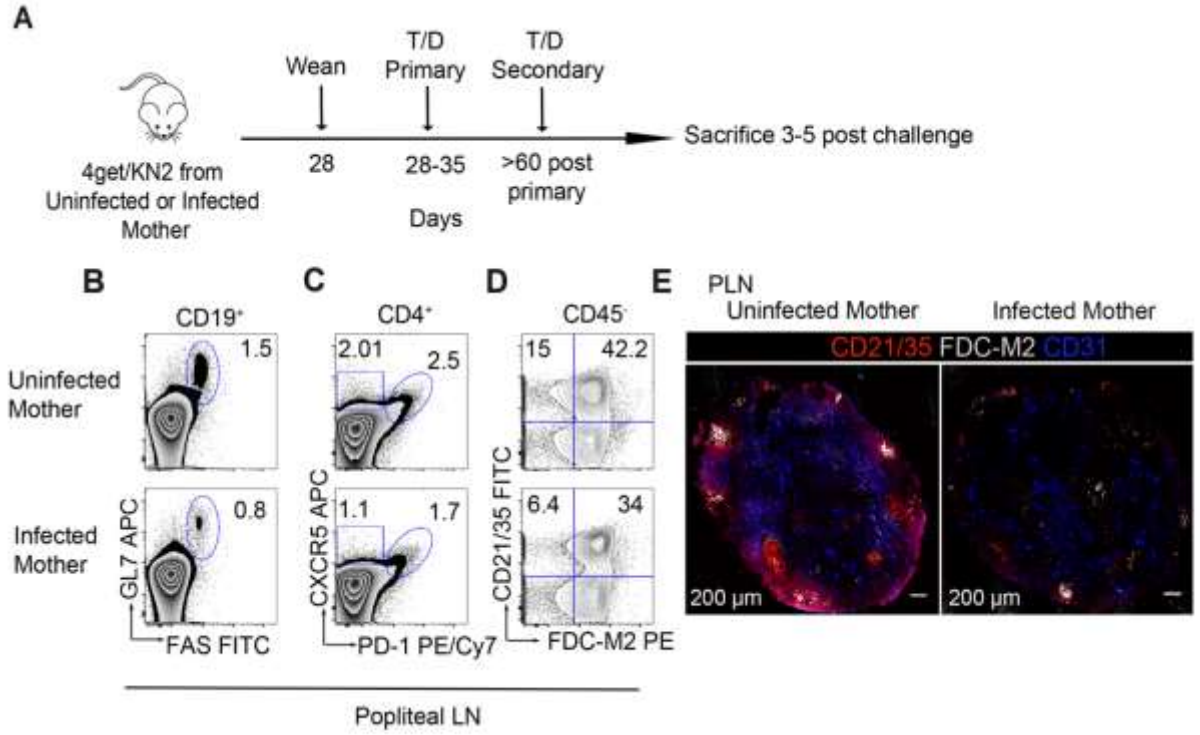


Figure 3.12. Memory B response is impaired recall immunization in offspring that were exposed to SEA antigens prenatally.

(A) Schematic of experimental timeline (B) Flow cytometry of GC B cells ($CD19^{+}GL\text{-}7^{+}FAS^{+}$), (C) Tfh ($PD\text{-}1^{+}CXCR5^{+}$, gated from $CD4^{+}$) (D) Follicular dendritic cells ($CD21/35^{+}FDC\text{-}M2^{+}CD45^{-}$) (E) Tile confocal imaging of draining lymph node in response to secondary immunization with aluminum Tetanus diphtheria, cryosections were stained for CD21/35 (red), FDC-M2 (gray) and CD31 (blue). Flow data are representative of n>4 mice per group and three independent experiments. Tile confocal imaging are representative of two independent experiments.

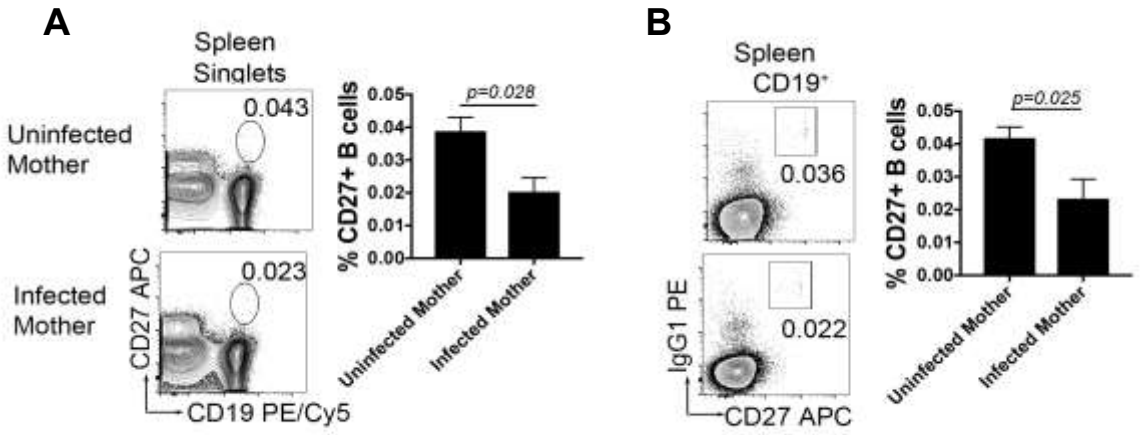


Figure 3.13 Memory B cells are required to maintain a secondary response to immunization.

(A) Bulk memory B cells ($CD19^{+}CD27^{+}$) and (B) IgG1 memory response ($IgG1^{+}CD27^{+}$)

CHAPTER 4. SCHISTOSOMA MANSONI INFECTION-INDUCED TRANSCRIPTIONAL CHANGES IN HEPATIC MACROPHAGE METABOLISM CORRELATE WITH AN ATHERO-PROTECTIVE PHENOTYPE

Modified from: Cortes-Selva D, Elvington AF, Ready A, Rajwa B, Pearce EJ, Randolph GJ and Fairfax KC (2018) Schistosoma mansoni Infection-Induced Transcriptional Changes in Hepatic Macrophage Metabolism Correlate with an Athero-Protective Phenotype. Front. Immunol. 9:2580. doi: 10.3389/fimmu.2018.02580

Introduction

Macrophages are highly plastic cells with vital functions in host defense and tissue repair and homeostasis (Sica & Mantovani, 2012). Their distinct functional characteristics depend on their distribution in different anatomical sites as well as the polarization signals from various tissue milieu (Gautier et al., 2012; Gordon, 2003; Gordon & Martinez, 2010). Due to their heterogeneity, a common classification of "classically activated" and "alternatively activated" has been attributed depending on their characteristic expression markers. While the classically activated macrophages (M1 macrophages) mediate autoimmunity and protection against different bacteria and viruses, alternatively activated macrophages (M2 macrophages) have been shown to play a role in wound healing, the regulation of inflammation, and metabolic functions (Leopold Wager & Wormley, 2014; Martinez & Gordon, 2014). Driven primarily by Toll-like receptor agonists and IFNγ, M1 polarized macrophages express inducible nitric oxide synthase (iNOS; *Nos2*) amongst other genes, and are pro-inflammatory, whereas M2 macrophages are induced by IL-4 and IL-13 and differentially express a range of genes including arginase-1 (*Arg1*), resistin like beta (Fizz-1; *Retnla*) and Ym1 (*Chil3*) (Murray & Wynn, 2011). M2 macrophages have previously been shown to play a role in both the defense against some parasitic infections, as well as the transition to

chronic helminth infection when immunomodulation and tissue repair is crucial for host survival (Gause, Wynn, & Allen, 2013). Importantly, they are the predominant macrophage in the liver during the Th2 dominated immune response to the helminth *Schistosoma mansoni*. In schistosomiasis, M2 macrophages are essential for survival of acute infection (Brunet, Finkelman, Cheever, Kopf, & Pearce, 1997; Herbert et al., 2004) and provide proline, a collagen precursor, while also aiding in both the development and modulation of fibrosis and liver pathology (Murray & Wynn, 2011). Recent studies have established that Arginase-1 producing M2 macrophages are essential for suppressing Th2 driven inflammation and fibrosis (Pesce et al., 2009), and our recent work identified immune complex-driven production of IL-10 by these macrophages as critical for immunomodulation in chronic infection (Fairfax et al., 2012). However, knowledge about the transcriptional profile of distinct macrophage subsets during schistosome infection remains limited.

Mice infected with *S. mansoni* serve as a reproducible model for human schistosomiasis. In both humans and mice, the pathology progresses from an early Th1 response into a Th2 dominated one in response to egg deposition in organs such as liver, intestines, and lungs by worm pairs. Eggs are highly immunogenic and toxic, and the host response to this stimulus leads to fibrosis and portal hypertension (Wynn, Thompson, Cheever, & Mentink-Kane, 2004). Previous studies have reported that schistosome infection in both humans and mice correlates with a significant modulation of lipid metabolism (Martins da Fonseca et al., 2014), lowering the total cholesterol levels of randomly bred mice and ApoE deficient mice (Stanley, Jackson, Griffiths, & Doenhoff, 2009), and reducing atherosclerotic lesions in ApoE deficient mice (Doenhoff, Stanley, Griffiths, & Jackson, 2002). Despite previous observations of the correlation of schistosome

infection and lipid metabolic changes in both human and murine models, the mechanism by which *S. mansoni* exerts protection against cardiovascular disease is not understood.

Here we describe microarray data of the transcriptional profiles of hepatic macrophages in naïve and *S. mansoni* infected mice that indicate a reduction in the transcriptional heterogeneity of both Kupffer cells and perivascular macrophages following schistosome infection that suggests a shared function for these distinct macrophage populations. Surprisingly, a large percentage of the transcriptional changes observed in macrophages relate to genes involved in cholesterol and phospholipid metabolism, including ApoC1. These data suggest a pivotal role of hepatic macrophages in the modulation of lipid metabolism during infection. Furthermore, we confirm the anti-atherogenic effect of active schistosome infection in an ApoE-/- atherosclerosis mouse model and postulate that this protection is due in part to significant reductions in serum ApoC1. Finally, we demonstrate that *S. mansoni* infection leads to a notable reduction in HFD induced insulin resistance and identify transcriptional changes to amino acid and glycogen metabolism that are likely mechanisms for this protection. Altogether, the data suggest an important and unexplored role of liver macrophages in the regulation of atherosclerosis and insulin sensitivity, a finding that has significant implications for diabetes, obesity and many other human diseases involving hyperlipidemia and insulin resistance

Results

S. mansoni infection profoundly alters hepatic macrophage metabolism

While it is widely accepted that hepatic macrophages play a critical role in the pathology and ultimately the survival of *S. mansoni* infection, the transcriptional changes that accompany the response to *S. mansoni* antigens are not entirely understood. We have previously established 10-weeks post-infection as a key time-point in immunomodulation where SEA (schistosome egg

antigen) specific B cells are recruited to the liver, and immune-complex ligation of hepatic macrophages begins, leading to macrophage IL-10 production (Fairfax et al., 2012). To explore the transcriptional changes to liver macrophages associated with *S. mansoni* infection, we first identified hepatic macrophages ($CD45^{+}PI^{-}Mertk^{+}CD64^{+}$) and then sorted $F4/80^{high}$ (Kupffer cells) and $F4/80^{int}$ (perivascular macrophages) populations from the livers of naïve and 10-week old *S. mansoni*-infected C57BL/6 mice according to Immgen standard protocols (Gautier et al., 2012; Heng, Painter, & Immunological Genome Project, 2008) (Figure 4.1). Cytospin examination of cells from both naïve and infected mice confirmed that both populations were macrophages, with the morphology of $F4/80^{high}$ macrophages displaying greater size heterogeneity than the $F4/80^{int}$ macrophages (Figure 4.1 B).

We compared the gene expression profile of naïve Kupffer cells and perivascular macrophages and found the expected markers for alternative activation (Figure 4.2 A) in addition to very high transcriptional diversity between these two macrophage populations considering that they reside in the same tissue environment (Figure 4.2 C). Subsequently, we performed the same comparison for 10-week post-infection samples and observed that transcript diversity decreases following *S. mansoni* infection (Figure 4.2 D), suggesting that Kupffer cells and perivascular macrophages may have shared functions in response to infection. Additionally, some of the transcripts that increased in both populations following infection (i.e. Ly6C) were consistent with both macrophage populations being repopulated by monocytes at this time-point, as recently described (Girgis et al., 2014; Nascimento et al., 2014). A recent report (Wang & Kubes, 2016) indicated that in models of sterile liver injury, peritoneal macrophages are recruited to the liver to aid in tissue repair, we did not, however, detect increases in Gata6 transcripts in our microarrays, so this paradigm may not apply to *S. mansoni* infection. We then performed an unsupervised

visualization of the gene expression profiles before and after *S. mansoni* infection via principal component analysis (Figure 4.3). The plot demonstrated that the PC 1 is associated with the difference between cells from the naive and the 10-week *S. mansoni*-infected animals, whereas the PC 2 described the difference between the F4/80^{high} and the F4/80^{int} cells regardless of the infection status. The ten genes with the highest contribution to PC 1 are Slc7a2, Timd4, Flrt2, F7, Atp6v0d2, Pdcd1lg2, Arg1, Ch25h, ApoC1, and Dhrs9. The ten genes with the highest contribution to PC 2 are Lum, Lox, Dpt, Cd163, Slc7a11, Igk-V1, Mgp, Jam2, Emcn, and Arhgap29.The effect sizes of the differences in the gene expressions represented as two-dimensional Mahalanobis distances in PC space are 18.83 (naïve F4/80^{int} cells vs. 10-week *S. mansoni* F4/80^{int} cells), 35.32 (naïve F4/80^{high} cells vs. 10-week *S. mansoni* F4/80^{high} cells). In both cases, the naïve vs. infected differences were statistically significant (Hoteling T^2 test, $p<10^{-5}$). Although distances for naïve F4/80^{high} vs. naïve F4/80^{int} cells and 10 -week infected F4/80^{high} vs. 10-week-infected F4/80^{low} cells were similar (4.40 and 4.09, respectively), the larger variance among naïve F4/80^{int} cells made the former pair of expressions indistinguishable in the PC space (Hoteling T^2 test, $p>0.1$). On the other hand, the dissimilarity between the gene expressions identified in the cells from the infected animals was significant ($p=0.004$).

Next, we employed the Boolean selection strategies detailed in the methods section to compare the transcriptional profiles of Kupffer cells and perivascular macrophages from 10-week infected animals to their naïve counterparts, and identified numerous subsets of differentially regulated genes (Figure 4.4). Subsets 3, 4, and 6 shed light on the putative functional roles of these two cell types in response to *S. mansoni* infection. Among 57 genes in the Subset 3 (q_hi∩hi_up∩int_down)\(q_int), we located Cbr2 (carbonyl reductase 2, involved in arachidonic acid metabolism), which is significantly ($p<0.05$) and highly (fold size=20.7) upregulated in

F4/80^{high}, but does not demonstrate a statistically significant change in F4/80^{int}, Alox ($p<0.05$, fold size=5.09), and F13a1 ($p<0.05$, fold size 20). Additionally, among 932 genes in Subset 4 (q_hi∩q_int∩int_down)\(hi_up), which is equivalent to (q_hi∩q_int∩int_down∩hi_down) and contains the genes which are significantly downregulated (foldsize <0) in both cell types we found Adh1, Alb, Aldh2, Apoa2, ApoB, ApoC1, ApoC3, Cd55, Clec4f, Creg1, Fabp7, Flt4, Gpr116, Hmcn1, Hpgd, Marco, Pde7b, Ptprb, Slc16a9, Slc40a1, Slco2a1, St3gal5, and Stab2. Surprisingly, this subset contains many genes involved in phospholipid and cholesterol metabolism, as well as solute transport. Further, we found that Prg4 and Spic belong to a Subset 5 of 795 genes that are downregulated in both macrophage types, but the downregulation in F4/80^{int} has not been statistically significant. The set is designated as (q_hi∩int_down)\(q_int∪hi_up). 683 genes were significantly simultaneously upregulated in F4/80^{int} and F4/80^{high} forming a Subset 6 (Figure 4.4 A), (q_hi∩q_int∩hi_up)\(int_down) = (q_hi∩q_int∩hi_up∩int_up). Among them we identified Anxa1, Arg,1 Atp1a3, Ccr2, Cd63, Chi3l3, Chi3l4, Dhrs9, Ear11, Egr2, Fcrlb, Fn1, Gda, Mgl2, Nfil3, Nos2, Pdcd1lg2, Retnla, Slc41a2, Slc7a2, and Uck2. This set contains many of the genes that are known to be hallmarks of alternative activation. Our filtering strategy identified 1167 genes belonging to (q_int∩hi_up)\(q_hi∪int_down), denoted as Subset 7. These genes demonstrate upregulation in both populations of macrophages; however, the upregulation in F4/80^{high} is not statistically significant. Among them, we found Ccl2, Ccl7, and Ptgs2. Another interesting subset of 357 genes (q_hi∩hi_up)\(q_int∪int_down) contained upregulated genes, which changed in a statistically significant manner in F4/80^{high} but not in F4/80^{int}. Ccl12 with a foldchange of ~15 and Ccl8 with a very high foldchange of 76.6 in F4/80^{high} as well as Mfsd6 were among these genes. Interestingly, there were only three genes in subset 1 (q_hi∩q_int∩hi_up∩int_down): Atrnl1, Mef2c, Nav1, and only 2 genes in subset 2

(q_hi∩q_int)\(hi_up∪int_down): Igf2bp2 and Ube2j2 (Figure 4.4), none of these genes had a particularly high absolute fold-change, further suggesting that both perivascular macrophages and Kupffer cells have a shared response to *S. mansoni* infection.

Some genes which were upregulated in both types of macrophages (or downregulated in both) nevertheless showed substantial differences in the level of regulation, exhibited by a large disparity in foldchanges. For instance, Rnase4 was upregulated in F4/80^{high} and F4/80^{int}; however, in the first case the observed foldchange was 11, and in the second instance is only 1.6. To further delineate key differential transcriptional changes, we used the absolute difference between log_2-foldchanges as a direct metric of differential regulation. The top 1-percentile of genes sorted by this measure contains 67 transcripts (Figure 4.4 C) expressed with log_2-foldchange difference ranging from 2.75 to 1.4. Among them, there are some of the known hallmarks of alternative activation (in the order of differences) Mgl2 (F4/80^{high} foldchange = 35.8, F4/80^{int} foldchange= 7.5), Retnla (F4/80^{high} foldchange = 84.2, F4/80^{int} foldchange=19.5) as well as Ear11, Chi3l3, Ptprb, Ccr2, Gda, Gpr116, St3gal5, Stab2, Cd63.

These data prompted us to conduct pathway analysis using the Advaita iPathwayGuide to uncover the pathways that are significantly manipulated by *S. mansoni* infection. Meta-analysis of transcriptional changes shared across both hepatic macrophage populations revealed striking alterations to multiple KEGG pathways, including multiple pathways of inflammation and cellular metabolism (Figure 4. 5 A), with the KEGG metabolic pathway showing the highest number of altered genes (Figure 4.5, pathway Bonferroni corrected p value=$1.042x10^{-8}$) We conclude that *S. mansoni* infection strongly alters the metabolic potential of liver macrophages. Since the liver is responsible for regulating many aspects of whole body metabolism (Langhans, 2003; Postic, Dentin, & Girard, 2004), these alterations may have profound effects on infected individuals.

Infection reduces ApoC1 production and aortic plaque formation

Schistosome infection in humans (Chen et al., 2013) and mice (Doenhoff et al., 2002; Hussaarts et al., 2015; Stanley et al., 2009) protects from the development of obesity and atherosclerosis, but the molecular mechanism(s) that underlies this protection has yet to be fully uncovered. The transcriptional profile revealed by our analysis suggested that *S. mansoni*-induced metabolic alterations to hepatic macrophages could be involved in infection-induced protection from metabolic diseases. To assess this hypothesis, we employed the $ApoE^{-/-}$ model of hyperlipidemia/atherosclerosis (Nakashima, Plump, Raines, Breslow, & Ross, 1994). $ApoE^{-/-}$ mice were fed either a high-fat Western diet (HFD) or normal chow diet for ten days before infection with *S. mansoni*, whereas controls were sham-infected. At 10-weeks post-infection we analyzed CD301 (galactose type C lectin) and CD206 (mannose receptor) expression as markers of alternative activation. Approximately 40-60% of macrophages from control chow uninfected were double positive, with the rest being $CD206^{+}$ (Figure 4.6). Infection increased the frequency of alternative activation to ~80% regardless of diet, suggesting that HFD do not interfere with *S. mansoni* induced macrophage polarization. Similar to previous reports (Doenhoff et al., 2002; Stanley et al., 2009), *S. mansoni*-infected mice on an HFD diet had significantly lower levels of plasma cholesterol and smaller aortic sinus plaques than uninfected HFD mice (Figure 4.7 A, B). Plaques in the aortic sinus of HFD infected mice also contained fewer $CD68^{+}$ macrophages (Figure 4.7 C), suggesting reduced recruitment of monocytes to the plaque, as these macrophages have been previously shown to be monocyte-derived (Swirski et al., 2006; Tacke et al., 2007). The transcriptional profile we uncovered in our analysis of schistosome-infected C57BL/6 liver macrophages (Figures 4.4, 4.5) showed reduced production of multiple apolipoproteins in macrophages, including ApoC1 (-10.00 log-FC, $p=1.35\text{x}10^{-5}$) and ApoC3 (-5.78 log-FC, $p=0.006$).

To confirm gene modulation on the ApoE$^{-/-}$ model, we assessed the expression of a list of genes of interest. Importantly, ApoC1 was significantly downregulated in the same manner in both the C57BL/6 and ApoE$^{-/-}$ model. Additionally, a previous report has indicated that ApoC1 production drives hyperlipidemia and pathogenesis in the ApoE$^{-/-}$ model (Westerterp, de Haan, Berbee, Havekes, & Rensen, 2006), making it a likely candidate for correlation with reduced plaque formation during infection. To address this possibility, we examined liver transcripts and plasma levels of ApoC1 and found that HFD induces an increase in ApoC1, but this increase is significantly attenuated by *S. mansoni* infection (Figure 4.7 D and E). This reduction, in systemic ApoC1 production, is a component of the postulated mechanism for *S. mansoni*-related protection from HFD- induced atherosclerosis.

Patent *S. mansoni* infection leads to improved glucose tolerance and alterations in hepatic macrophage amino acid biosynthesis and glucagon signaling pathways

Schistosomiasis has been reported to correlate with lower hemoglobin A1c levels in populations with a previous history of infection (Chen et al., 2013), as well in a murine model of obesity (Hussaarts et al., 2015), so we sought to examine glucose tolerance in this model. *S. mansoni* induced reductions in serum cholesterol have previously been shown to be egg dependent, so we assessed glucose sensitivity in a 2-hour glucose tolerance test (GTT) at 5-weeks post infection (pre-egg laying). We found that pre-patency, *S. mansoni* infection has no significant effect on glucose tolerance in normal chow fed mice as measured by area under the glucose excursion curve (AUC, Figure 4.8). HFD increases blood glucose levels and decreases glucose tolerance (Figure 4.8 A), while pre-patent *S. mansoni* infection has no significant effect on this increase. At the transition to chronic infection (10 weeks post infection), normal chow fed infected mice have lower fasting glucose levels and significantly improved whole-body glucose tolerance

(Figure 4.8). HFD significantly impairs glucose tolerance (as compared to chow uninfected controls), while *S. mansoni* infection of HFD fed mice restores glucose tolerance to a similar level as chow uninfected mice (Figure 4.8). This is distinct from what has previously been reported, where *S. mansoni* infection of C57BL/6 mice improved glucose tolerance in HFD fed mice, but not normal low-fat chow fed mice. This difference suggests that there may be strain-specific differences in the control of glucose sensitivity. Since $ApoE^{-/-}$ are prone to develop hyperlipidemia and atherosclerosis spontaneously as they age, it is possible that this propensity underlies the difference.

We then went back to the microarray pathway analysis and looked for pathway changes that could be expected to lead to improvement in glucose tolerance based on published reports. We found that *S. mansoni* infection significantly alters the biosynthesis of amino acids in Kupffer cells (Figure 4.8, pathway analysis Bonferroni corrected p value= 0.006), and one of the genes in that pathway, Psat1, stood out as a candidate for involvement in improving glucose tolerance as its transcript level is increased in infected C57BL/6 mice (logFC= 5,). The correlation between modulation of this transcript and infection was confirmed in our $ApoE^{-/-}$ model (Figure 4.8 C). Hepatic psat1 transcript levels (measured in total liver RNA) have previously been shown to be reduced in both genetic and diet-induced models of diabetes, while global overexpression of PSAT1 improves insulin signaling and sensitivity (Yu et al., 2015). The glucagon signaling pathway is also significantly altered (Figure 4.8 D, pathway Bonferroni corrected p value= 0.015), in that pathway, *S. mansoni* infection decreases pygl expression while also increasing gys1 transcripts. Genetic disruption of PYGL has previously been shown to improve glucose tolerance *in vivo* (Kelsall, Rosenzweig, & Cohen, 2009). While GYS1 is one of two enzymes responsible for glycogen synthesis, and small molecule induced activation of GYS1 in diabetic mice improves

glucose tolerance (Nakano et al., 2017). The combined effects of these two alterations would be predicted to lead to increased glucose tolerance and insulin sensitivity. We then asked whether gys1 transcripts are similarly altered by *S. mansoni* infection of HFD fed ApoE$^{-/-}$ mice. *S. mansoni* infection leads to significantly higher gys1 transcripts in the liver tissue of both chow and HFD fed mice (Figure 4.8 E). Psat1 is similarly altered by infection (Figure 4.8 F), with transcripts significantly higher in *S. mansoni* infected mice on both chow and HFD, although transcript levels of HFD infected mice are lower than those of chow infected. These data suggest that the *S. mansoni* induced transcriptional changes that were identified via the microarray also occur in the liver tissue of HFD fed ApoE$^{-/-}$ mice, supporting the hypothesis that *S. mansoni* induced alterations in hepatic macrophage metabolism are able to influence whole body metabolism and help protect from pathological changes generally associated with HFD induced metabolic disorders.

Discussion

Overall, our data provide the first complete transcriptional analysis of Kupffer cells and perivascular macrophages in steady state and chronic *S. mansoni* infection. These data suggest that during *S. mansoni* infection, egg-induced M2 macrophages demonstrate profound alterations to their metabolic profile, with shifts in the balance of phospholipid and glucose metabolism as well as increased amino acid metabolism, and that these changes correlate with improvements in whole body cholesterol and glucose metabolism. While many of these alterations are shared between both perivascular macrophages and Kupffer cells, there are key genes that are differentially regulated in the two populations. Cbr2, Alox5, and F13a1 are all significantly up-regulated in Kupffer cells, but not in perivascular macrophages. Since Cbr2 and Alox are involved in arachidonic acid metabolism, it suggests that while both populations are alternatively activated, Kupffer cells specifically increase leukotriene production in the granuloma. Similarly, F13a1 has been

previously implicated in wound healing (Andersson et al., 2015), suggesting the Kupffer cells are more involved in the process of granuloma formation and resolution. Conversely, Lox is upregulated in both macrophage populations, but the increase in perivascular macrophages is far higher (8.3-fold vs. 2.1-fold), suggesting that perivascular macrophages are responding to greater oxidative stress during *S. mansoni* infection, a proposition that is supported by their tissue location. Many of the key markers of M2 activation (Chi3l3, Retnal, Mgl2) have a lower fold upregulation in perivascular macrophages, a finding that is matched by the frequency of these macrophages that are $CD206^{+}CD301^{+}$, suggesting that there may be degrees of alternative activation in the liver in the context of *S. mansoni* infection. The fact that only 5 genes (Subsets 1 and 2) are truly differentially regulated (opposite signs of significant fold changes) supports the hypothesis that after infection Kupffer cells and Perivascular macrophages act to a large degree in unison.

Recent work, using other models of M2 macrophage generation, have indicated that M2 macrophages rely on lipolysis driven fatty acid oxidation (Huang et al., 2014; Vats et al., 2006) for the generation and maintenance of the M2 state. Our transcriptional analysis of Kupffer cells and perivascular macrophages suggest similar alterations are induced by chronic *S. mansoni* infection, but our data also provide the first evidence that alterations to hepatic macrophage metabolism either reflect or influence infection-induced alterations to whole body metabolism. Macrophages are known to be critical sources of apolipoproteins like ApoC1 (Westerterp et al., 2007; Westerterp et al., 2006), and LPS-induced inflammation has been linked with increased levels of plasma cholesterol and ApoC1 (Westerterp et al., 2007). Our data indicating that *S. mansoni*-induced M2 transcriptional changes reduce both ApoC1 production and plasma cholesterol is in concordance with the established paradigm of M1 and M2 polarization states as driving opposite pathological states, and with the hypothesis that M2 macrophages play a role in

the resolution and modulation of atherosclerotic lesions (Chinetti-Gbaguidi et al., 2011; Chinetti-Gbaguidi, Colin, & Staels, 2015; Roma-Lavisse et al., 2015).

The liver plays a critical role in whole body metabolism through its control of glycogen metabolism, with glycogen synthase (GYS1) and glycogen phosphorylase (PYGL) acting to respectively increase and decrease stored glycogen to maintain blood sugar homeostasis. Our pathway analysis revealed key alterations to both macrophage amino acid and glucagon signaling/metabolic pathways. We identified key genes (GYS1, PYGL, and PSAT1) from both pathways that have previously been demonstrated to play a role in glucose tolerance/ insulin sensitivity in models of obesity and diabetes (Gasa, Clark, Yang, DePaoli-Roach, & Newgard, 2002; Yu et al., 2015) and epidemiological studies (Schalin-Jantti, Harkonen, & Groop, 1992; Shimomura et al., 1997; St-Onge, Joanisse, & Simoneau, 2001). We hypothesize that the combined effects of *S. mansoni*-induced alterations to these three genes are at least partially responsible for infection-related improvements in glucose tolerance in both chow and HFD fed mice, a proposition that will be tested in future studies. These data raise the possibility that *S. mansoni*-induced polarization of liver macrophages can significantly alter whole body metabolism. While multiple previous studies in humans (Aravindhan et al., 2010; Chen et al., 2013; Wiria et al., 2015) and mice (Doenhoff et al., 2002; Hussaarts et al., 2015; Stanley et al., 2009) have indicated that helminth infections in general, and schistosomes in specific, modulate susceptibility to the development of metabolic diseases, a molecular mechanism underlying this modulation has not been identified. We postulate that *S. mansoni*-induced M2 polarization of hepatic macrophages are involved in protection from HFD induced hyperlipidemia, atherosclerosis, and glucose intolerance. Future studies will focus on thoroughly understanding the infection-induced transcriptional changes to other tissue macrophage populations and the broader monocyte lineage.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committees of Washington University in St. Louis and Purdue University.

Mice and Parasites

Male C57BL/6J mice were purchased from Jackson Laboratories, *ApoE*$^{-/-}$ (B6.129P2-Apoetm1Unc/J) mice were bred at Purdue University. Mice were kept under specific pathogen–free conditions and male mice were infected at 6 weeks of age. Snails infected with *S. mansoni* (strain NMRI, NR-21962) were provided by the Schistosome Research Reagent Resource Center for distribution by BEI Resources, NIAID NIH. Mice were exposed percutaneously to 90 *Schistosoma mansoni* cercariae. For experiments using *Apoe*$^{-/-}$ mice, animals were transitioned to HFD (21% milk fat, 0.15% cholesterol; TD 88137 Harlan Teklad) 10 days prior to *S. mansoni* infection. Control mice were fed standard rodent chow (2018 rodent chow, Harlan Teklad) and mice were sacrificed at 10-weeks post-infection.

Macrophage identification and Isolation

Livers were removed from PBS-perfused animals, mashed, and incubated in RPMI (Mediatech) containing 250 μg/ml Collagenase D (Roche) at 37°C for 60 min. The resulting suspension was disrupted through a 100 μm metal cell strainer, washed, and then red blood cell lysed with ACK lysis buffer (BD) two times to remove hepatocytes followed by washing. The resulting pellet was washed and used for sorting. Surface staining with monoclonal antibodies,

acquisition, and sorting were performed according to Immgen standard protocols (www.immgen.org). The following mAb (eBioscience, BioLegend, or R&D systems) against mouse antigens were used as PE, PE-Cy5, PE-Cy7, allophycocyanin (APC), APC-Cy7, Pacific blue, or biotin conjugates: CD11c (N418), CD11b (M1/70), CD45 (30-F11), MHC-II (M5/114.15.2), F4/80 (BM8), and MERTK (BAF591). Cells were directly sorted from mouse tissues and were processed from tissue procurement to a second round of sorting into TRizol within 4 h using a Beckton-Dickinson Aria II instrument.

Microarray analysis, normalization, and dataset analysis

RNA was amplified and hybridized on the Affymetrix Mouse Gene 1.0 ST array by the Immgen consortium using double-sorted cell populations sorted directly into TRIzol. Data analysis utilized GenePattern analysis software. Raw data were normalized using the robust multi-array algorithm, returning linear values between 10 to 20,000. A common threshold for positive expression at 95% confidence across the dataset was determined to be 120. Differential gene expression signatures were identified and visualized using the "Multiplot" module of GenePattern (http://www.broadinstitute.org/cancer/software/genepattern/). Probesets were considered as differentially expressed with a coefficient of variation less than 0.5 and a p-value < 0.05 (Student's T-test). Calculation and visualization of differential gene expression sizes (calculated as $\log_2$-transformed Hedges' g measures accompanied by 95% confidence intervals and t-test p-values indicating significance of differences, raw foldchanges, and $\log_2$-foldchanges) were performed using R-language for statistical computing.

To identify important genes (i.e., the genes expressed in a matter suggesting a link to macrophage class and/or infection status) we used various Boolean selection strategies focusing on multiple scenarios:

1. Genes which are upregulated in F4/80^{high} (Kupffer cells) (foldchange > 0), but downregulated in F4/80^{int} (perivascular macrophages) (foldchange <0).
2. Genes which are downregulated in F4/80^{high} (foldchange <0), but upregulated in F4/80^{int} (foldchange > 0)
3. Genes which are significantly (Benjamini-Hochberg adjusted $p<0.05$) upregulated in both cell types
4. Genes which are significantly (Benjamini-Hochberg adjusted $p<0.05$) downregulated in both cell type
5. Genes which differ substantially in the degree of regulation (regardless of the direction of regulation).

The described Boolean strategies are illustrated in the Venn diagrams in Figure 2A-C. The following set designations were used in the set notation:

- q_hi = Significantly regulated genes in F4/80^{high} cells (BH adjusted $p<0.05$)
- q_nt = Significantly regulated genes in F4/80^{int} cells (BH adjusted $p<0.05$)
- hi_up = Genes upregulated in F4/80^{high} cells
- int_up = Genes upregulated genes in F4/80^{int} cells
- hi_down = Genes downregulated in F4/80^{high} cells
- int_down = Genes downregulated in F4/80^{int} cells

The employed set notation:

- $A \cap B$ – A intersect B
- $A \cup B$ – A union B
- $A \setminus B$ – A minus B, or A complement B

The Data (significantly impacted pathways, biological processes, molecular interactions.) were analyzed using Advaita Bio's iPathwayGuide (http://www.advaitabio.com/ipathwayguide). Pathway analysis was performed on log_2-transformed data using Bonferroni-corrected p-values. Data are deposited in GenBank under accession no. GSE37448 as part of the Immgen 2 dataset.

Principle Component Analysis

For unsupervised visualization of the gene expression profiles via principal component analysis, the gene expression data were constrained to a simplex and transformed using centered log-ratio transformation. The resultant values were ordered according to their variances, and the top 0.5-percentile of the genes were selected for the computation of the principal components (PCs)

Flow cytometric analysis

Livers were perfused with 1X PBS, mashed and digested in DMEM containing 250 µg/ml Collagenase (Sigma) at 37^0C for 30 minutes. The digested livers were then mashed and filtered through a 100 µm metal strainer and digestion was repeated for 15 minutes. Total liver contents were strained and washed with DMEM. The pellet was lysed with 1X lysis buffer (BD PharmLyse), quenched, and washed. The resulting cell suspension was used in flow cytometry. Surface staining was performed using the following mAb against mouse antigens: CD45 (30-F11, eBioscience), CD301(BioRad), CD206 (C068C2, Biolegend), F4/80 (BM8, Biolegend), mouse Mer biotinylated (R&D), CD64(X54-5/7.1, BD). Samples were acquired using FACSCanto II flow cytometer (BD) and analyzed using Flowjo X 10.0.7r2 (FlowJo LLC, Inc).

Analysis of atherosclerotic plaques.

At sacrifice, mice were perfused with PBS, and the heart was extracted for plaque assessment at the aortic sinus. Following fixation in 4% paraformaldehyde, tissue was cryoprotected in 30% sucrose, embedded in OCT compound (Fisher Scientific), and flash frozen. Fifteen-micron cryosections were made in the aortic sinus from initiation to termination of the aortic valve leaflets. Plaque assessment in the aortic sinus was performed as previously described (Potteaux et al., 2011), and is briefly described as follows. The total plaque was determined by taking the average of total atherosclerotic plaque from at least five sections at least 60 microns apart. Macrophage content was determined using CD68 immunofluorescence: average CD68 (BioRad) positive area was assessed from at least five sections at least 60 microns apart. Quantification of plaque area and area occupied by CD68 was calculated with ImageJ software.

Cholesterol Quantitation

Total cholesterol was measured from plasma as previously described (Potteaux et al., 2011) using colorimetric assessment methods. In brief, whole blood was collected by mandibular bleed into EDTA-plasma collection tubes, and the plasma fraction was removed following red blood cell sedimentation via centrifugation. Plasma samples were stored at -80 °C until use. Total plasma cholesterol was assessed by manufacturer's protocol (Wako Diagnostics, #999-02601). Values were calculated from the mean of two replicates from a standard calibration curve. Total plasma cholesterol was reported as mg per dL.

RNA isolation and q-RT-PCR analysis

Sections of perfused livers were homogenized in Trizol, and RNA isolation was performed as previously described (Immunological Genome Project, Total RNA isolation with Trizol

(Fairfax, Everts, Smith, & Pearce, 2013)). RNA was used for cDNA synthesis using Superscript II (Invitrogen) for qPCR analysis. qPCR was performed using TaqMan Gene expression assays (gys1, CD36, ApoC1, ThermoFisher) or SYBR Green (psat1(Yu et al., 2015), primers forward: ACG CCA AAG GAG ACG AAG CT and reverse: ATG TTG AGT TCT ACC GCC TTG TC) on an Applied Biosystems Stepone Plus Real-Time PCR System. Beta-Actin assay number Mm00607939_s1, GYS1 assay Mm01962575_s1, CD36 assay Mm00432403_m1, ApoC1 assay Mm00431816_m1. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

ELISA

ApoC1 plasma concentrations were determined by ELISA using an anti-mouse ApoC1 ELISA kit as per the manufacturer's instructions (LSBio).

Statistics

Statistical analyses for non-microarray data were performed using one-way ANOVA, a non-parametric Mann-Whitney test, or unpaired Student's t-test depending on the distribution of the data. P-values ≤ 0.05 were considered statistically significant. Graph generation and statistical analyses were performed using Prism (GraphPad v7.0) and R-language for statistical computing.

References

Andersson, C., Kvist, P. H., McElhinney, K., Baylis, R., Gram, L. K., Pelzer, H., . . . Palumbo, J. S. (2015). Factor XIII Transglutaminase Supports the Resolution of Mucosal Damage in Experimental Colitis. PLoS One, 10(6), e0128113. doi:10.1371/journal.pone.0128113

Aravindhan, V., Mohan, V., Surendar, J., Muralidhara Rao, M., Pavankumar, N., Deepa, M., . . . Babu, S. (2010). Decreased prevalence of lymphatic filariasis among diabetic subjects associated with a diminished pro-inflammatory cytokine response (CURES 83). PLoS Negl Trop Dis, 4(6), e707. doi:10.1371/journal.pntd.0000707

Brunet, L. R., Finkelman, F. D., Cheever, A. W., Kopf, M. A., & Pearce, E. J. (1997). IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. J Immunol, 159(2), 777-785.

Chen, Y., Lu, J., Huang, Y., Wang, T., Xu, Y., Xu, M., . . . Ning, G. (2013). Association of previous schistosome infection with diabetes and metabolic syndrome: a cross-sectional study in rural China. J Clin Endocrinol Metab, 98(2), E283-287. doi:10.1210/jc.2012-2517

Chinetti-Gbaguidi, G., Baron, M., Bouhlel, M. A., Vanhoutte, J., Copin, C., Sebti, Y., . . . Staels, B. (2011). Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARgamma and LXRalpha pathways. Circ Res, 108(8), 985-995. doi:10.1161/CIRCRESAHA.110.233775

Chinetti-Gbaguidi, G., Colin, S., & Staels, B. (2015). Macrophage subsets in atherosclerosis. Nat Rev Cardiol, 12(1), 10-17. doi:10.1038/nrcardio.2014.173

Doenhoff, M. J., Stanley, R. G., Griffiths, K., & Jackson, C. L. (2002). An anti-atherogenic effect of Schistosoma mansoni infections in mice associated with a parasite-induced lowering of blood total cholesterol. Parasitology, 125(Pt 5), 415-421.

Fairfax, K. C., Amiel, E., King, I. L., Freitas, T. C., Mohrs, M., & Pearce, E. J. (2012). IL-10R blockade during chronic schistosomiasis mansoni results in the loss of B cells from the liver and the development of severe pulmonary disease. PLoS Pathog, 8(1), e1002490. doi:10.1371/journal.ppat.1002490

Fairfax, K. C., Everts, B., Smith, A. M., & Pearce, E. J. (2013). Regulation of the Development of the Hepatic B Cell Compartment during Schistosoma mansoni Infection. J Immunol. doi:10.4049/jimmunol.1301357

Gasa, R., Clark, C., Yang, R., DePaoli-Roach, A. A., & Newgard, C. B. (2002). Reversal of diet-induced glucose intolerance by hepatic expression of a variant glycogen-targeting subunit of protein phosphatase-1. J Biol Chem, 277(2), 1524-1530. doi:10.1074/jbc.M107744200

Gause, W. C., Wynn, T. A., & Allen, J. E. (2013). Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths. Nat Rev Immunol, 13(8), 607-614. doi:10.1038/nri3476

Gautier, E. L., Shay, T., Miller, J., Greter, M., Jakubzick, C., Ivanov, S., . . . Immunological Genome, C. (2012). Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. Nat Immunol, 13(11), 1118-1128. doi:10.1038/ni.2419

Girgis, N. M., Gundra, U. M., Ward, L. N., Cabrera, M., Frevert, U., & Loke, P. (2014). Ly6C(high) monocytes become alternatively activated macrophages in schistosome granulomas with help from CD4+ cells. PLoS Pathog, 10(6), e1004080. doi:10.1371/journal.ppat.1004080

Gordon, S. (2003). Alternative activation of macrophages. Nat Rev Immunol, 3(1), 23-35. doi:10.1038/nri978

Gordon, S., & Martinez, F. O. (2010). Alternative activation of macrophages: mechanism and functions. Immunity, 32(5), 593-604. doi:10.1016/j.immuni.2010.05.007

Heng, T. S., Painter, M. W., & Immunological Genome Project, C. (2008). The Immunological Genome Project: networks of gene expression in immune cells. Nat Immunol, 9(10), 1091-1094. doi:10.1038/ni1008-1091

Herbert, D. R., Holscher, C., Mohrs, M., Arendse, B., Schwegmann, A., Radwanska, M., . . . Brombacher, F. (2004). Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. Immunity, 20(5), 623-635.

Huang, S. C., Everts, B., Ivanova, Y., O'Sullivan, D., Nascimento, M., Smith, A. M., . . . Pearce, E. J. (2014). Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. Nat Immunol, 15(9), 846-855. doi:10.1038/ni.2956

Hussaarts, L., Garcia-Tardon, N., van Beek, L., Heemskerk, M. M., Haeberlein, S., van der Zon, G. C., . . . Guigas, B. (2015). Chronic helminth infection and helminth-derived egg antigens promote adipose tissue M2 macrophages and improve insulin sensitivity in obese mice. FASEB J, 29(7), 3027-3039. doi:10.1096/fj.14-266239

Kelsall, I. R., Rosenzweig, D., & Cohen, P. T. (2009). Disruption of the allosteric phosphorylase a regulation of the hepatic glycogen-targeted protein phosphatase 1 improves glucose tolerance in vivo. Cell Signal, 21(7), 1123-1134. doi:10.1016/j.cellsig.2009.03.001

Langhans, W. (2003). Role of the liver in the control of glucose-lipid utilization and body weight. Curr Opin Clin Nutr Metab Care, 6(4), 449-455. doi:10.1097/01.mco.0000078993.96795.16

Leopold Wager, C. M., & Wormley, F. L., Jr. (2014). Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal infections. Mucosal Immunol, 7(5), 1023-1035. doi:10.1038/mi.2014.65

Martinez, F. O., & Gordon, S. (2014). The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep, 6, 13. doi:10.12703/P6-13

Martins da Fonseca, C. S., Pimenta Filho, A. A., dos Santos, B. S., da Silva, C. A., Domingues, A. L., Owen, J. S., & Lima, V. L. (2014). Human plasma lipid modulation in schistosomiasis mansoni depends on apolipoprotein E polymorphism. PLoS One, 9(7), e101964. doi:10.1371/journal.pone.0101964

Murray, P. J., & Wynn, T. A. (2011). Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol, 11(11), 723-737. doi:10.1038/nri3073

Nakano, K., Takeshita, S., Kawasaki, N., Miyanaga, W., Okamatsu, Y., Dohi, M., & Nakagawa, T. (2017). AJS1669, a novel small-molecule muscle glycogen synthase activator, improves glucose metabolism and reduces body fat mass in mice. Int J Mol Med, 39(4), 841-850. doi:10.3892/ijmm.2017.2909

Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L., & Ross, R. (1994). ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. Arterioscler Thromb, 14(1), 133-140.

Nascimento, M., Huang, S. C., Smith, A., Everts, B., Lam, W., Bassity, E., . . . Pearce, E. J. (2014). Ly6Chi monocyte recruitment is responsible for Th2 associated host-protective macrophage accumulation in liver inflammation due to schistosomiasis. PLoS Pathog, 10(8), e1004282. doi:10.1371/journal.ppat.1004282

Pesce, J. T., Ramalingam, T. R., Mentink-Kane, M. M., Wilson, M. S., El Kasmi, K. C., Smith, A. M., . . . Wynn, T. A. (2009). Arginase-1-expressing macrophages suppress Th2 cytokine-driven inflammation and fibrosis. PLoS Pathog, 5(4), e1000371. doi:10.1371/journal.ppat.1000371

Postic, C., Dentin, R., & Girard, J. (2004). Role of the liver in the control of carbohydrate and lipid homeostasis. Diabetes Metab, 30(5), 398-408.

Potteaux, S., Gautier, E. L., Hutchison, S. B., van Rooijen, N., Rader, D. J., Thomas, M. J., . . . Randolph, G. J. (2011). Suppressed monocyte recruitment drives macrophage removal from atherosclerotic plaques of Apoe-/- mice during disease regression. J Clin Invest, 121(5), 2025-2036. doi:10.1172/JCI43802

Roma-Lavisse, C., Tagzirt, M., Zawadzki, C., Lorenzi, R., Vincentelli, A., Haulon, S., . . . Dupont, A. (2015). M1 and M2 macrophage proteolytic and angiogenic profile analysis in atherosclerotic patients reveals a distinctive profile in type 2 diabetes. Diab Vasc Dis Res, 12(4), 279-289. doi:10.1177/1479164115582351

Schalin-Jantti, C., Harkonen, M., & Groop, L. C. (1992). Impaired activation of glycogen synthase in people at increased risk for developing NIDDM. Diabetes, 41(5), 598-604.

Shimomura, H., Sanke, T., Ueda, K., Hanabusa, T., Sakagashira, S., & Nanjo, K. (1997). A missense mutation of the muscle glycogen synthase gene (M416V) is associated with insulin resistance in the Japanese population. Diabetologia, 40(8), 947-952. doi:10.1007/s001250050772

Sica, A., & Mantovani, A. (2012). Macrophage plasticity and polarization: in vivo veritas. J Clin Invest, 122(3), 787-795. doi:10.1172/JCI59643

St-Onge, J., Joanisse, D. R., & Simoneau, J. A. (2001). The stimulation-induced increase in skeletal muscle glycogen synthase content is impaired in carriers of the glycogen synthase XbaI gene polymorphism. Diabetes, 50(1), 195-198.

Stanley, R. G., Jackson, C. L., Griffiths, K., & Doenhoff, M. J. (2009). Effects of Schistosoma mansoni worms and eggs on circulating cholesterol and liver lipids in mice. Atherosclerosis, 207(1), 131-138. doi:10.1016/j.atherosclerosis.2009.04.037

Swirski, F. K., Pittet, M. J., Kircher, M. F., Aikawa, E., Jaffer, F. A., Libby, P., & Weissleder, R. (2006). Monocyte accumulation in mouse atherogenesis is progressive and proportional to extent of disease. Proc Natl Acad Sci U S A, 103(27), 10340-10345. doi:10.1073/pnas.0604260103

Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., . . . Randolph, G. J. (2007). Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. J Clin Invest, 117(1), 185-194. doi:10.1172/JCI28549

Vats, D., Mukundan, L., Odegaard, J. I., Zhang, L., Smith, K. L., Morel, C. R., . . . Chawla, A. (2006). Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. Cell Metab, 4(1), 13-24. doi:10.1016/j.cmet.2006.05.011

Wang, J., & Kubes, P. (2016). A Reservoir of Mature Cavity Macrophages that Can Rapidly Invade Visceral Organs to Affect Tissue Repair. Cell, 165(3), 668-678. doi:10.1016/j.cell.2016.03.009

Westerterp, M., Berbee, J. F., Pires, N. M., van Mierlo, G. J., Kleemann, R., Romijn, J. A., . . . Rensen, P. C. (2007). Apolipoprotein C-I is crucially involved in lipopolysaccharide-induced atherosclerosis development in apolipoprotein E-knockout mice. Circulation, 116(19), 2173-2181. doi:10.1161/CIRCULATIONAHA.107.693382

Westerterp, M., de Haan, W., Berbee, J. F., Havekes, L. M., & Rensen, P. C. (2006). Endogenous apoC-I increases hyperlipidemia in apoE-knockout mice by stimulating VLDL production and inhibiting LPL. J Lipid Res, 47(6), 1203-1211. doi:10.1194/jlr.M500434-JLR200

Wiria, A. E., Hamid, F., Wammes, L. J., Prasetyani, M. A., Dekkers, O. M., May, L., . . . Smit, J. W. (2015). Infection with Soil-Transmitted Helminths Is Associated with Increased Insulin Sensitivity. PLoS One, 10(6), e0127746. doi:10.1371/journal.pone.0127746

Wynn, T. A., Thompson, R. W., Cheever, A. W., & Mentink-Kane, M. M. (2004). Immunopathogenesis of schistosomiasis. Immunol Rev, 201, 156-167. doi:10.1111/j.0105-2896.2004.00176.x

Yu, J., Xiao, F., Guo, Y., Deng, J., Liu, B., Zhang, Q., . . . Guo, F. (2015). Hepatic Phosphoserine Aminotransferase 1 Regulates Insulin Sensitivity in Mice via Tribbles Homolog 3. Diabetes, 64(5), 1591-1602. doi:10.2337/db14-1368

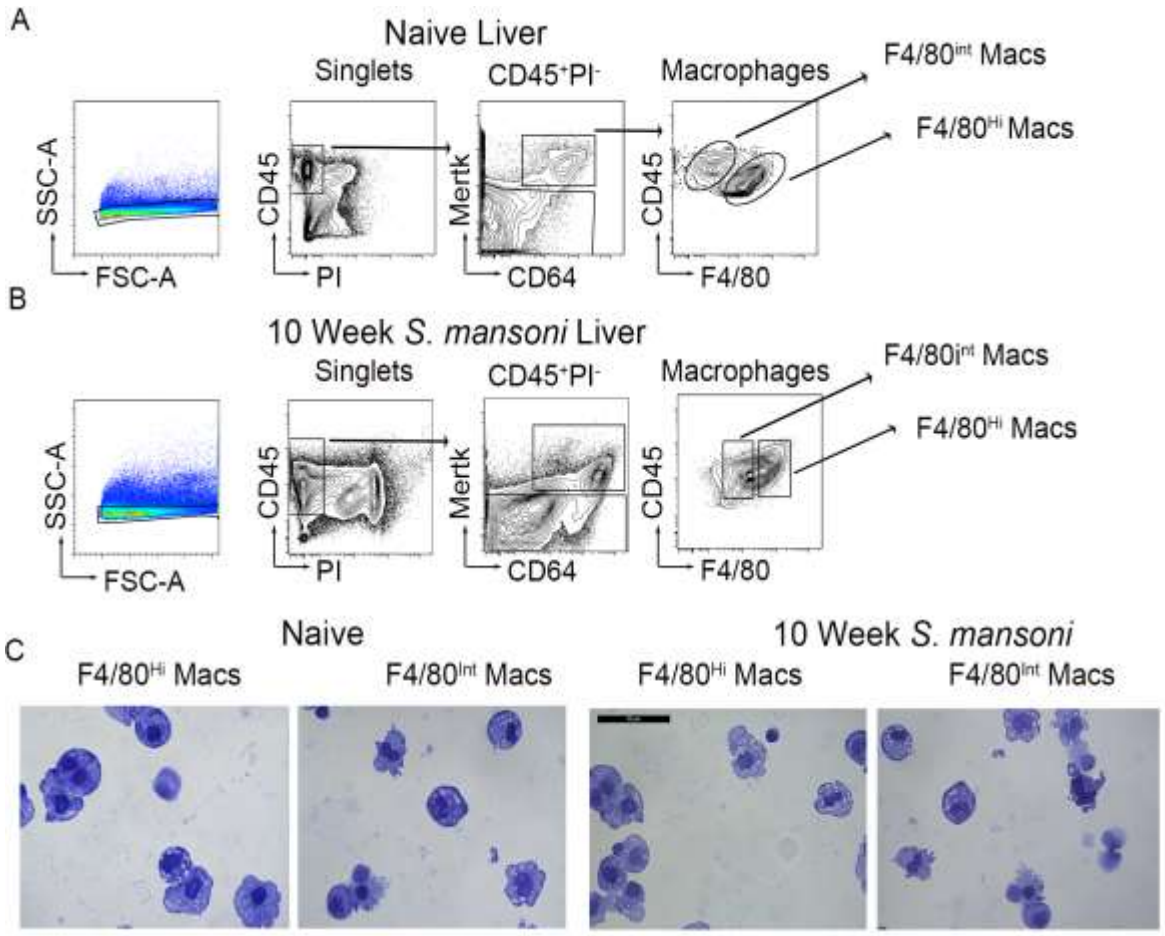


Figure 4.1. Murine livers contain two distinct macrophage populations.

C57BL/6 male mice were either naïve or infected with *S. mansoni* for the indicated number of weeks. (A-B) Perfused livers were removed, digested with collagenase for 1 hour, and disrupted through a metal strainer to create single cell suspensions. Cells were identified by staining for the indicated markers and sorted on an Aria II. All populations were sorted two times, with the second sort going directly into Trizol according to the Immgen protocol. Individual animals were sorted from multiple litters/infections to achieve 4-5 biological replicates for individual microarray analysis. (C) Representative cytospins of aliquots from the second sorts of each macrophage populations were stained with H&E.

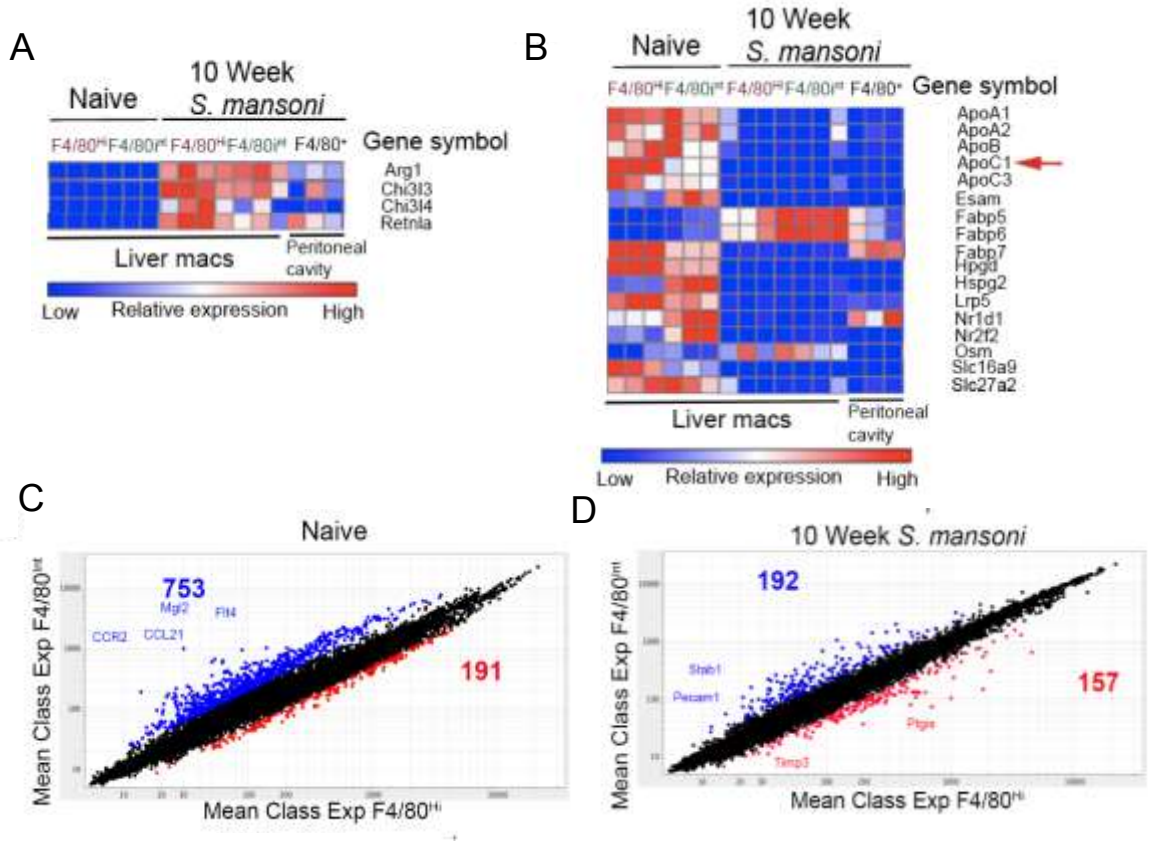


Figure 4.2 *S. mansoni* infection decrease transcript diversity between Kupffer cells and perivascular macrophages.

Total RNA was extracted, amplified, and transcript expression determined using whole-mouse genome Affymetrix Mouse Gene 2.0 ST Arrays through the Immunological genome consortium. (A) Microarray of relative expression of markers of infection (B) Microarray of differentially expressed genes in steady state and following infection (C) The number of probes increased by 2-fold with a p value > 0.05 Blue indicates increased expression in F4/80^{int} liver macrophages at steady state, Red indicates increased expression in F4/80^{hi} liver macrophages at steady state (D) The number of probes increased by 2-fold with a p value > 0.05 Blue indicates increased expression in F4/80^{int} liver macrophages at steady state, Red indicates increased expression in F4/80^{hi} liver macrophages at 10-weeks post *S. mansoni* infection.

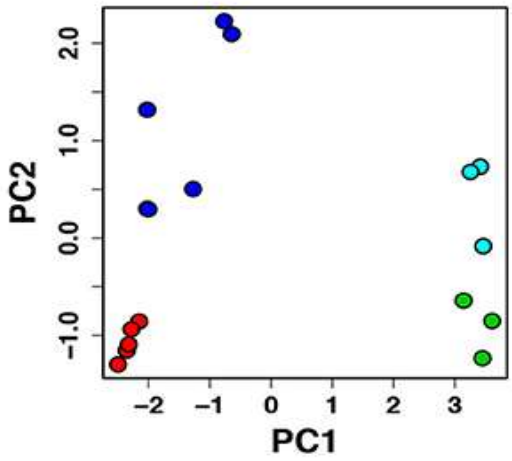


Figure 4.3 Principal component analysis of genes associated with the difference from the naive and the 10-week *S. mansoni*-infected animals and the difference between the F4/80^{high} and the F4/80^{int} cells regardless of the infection status.

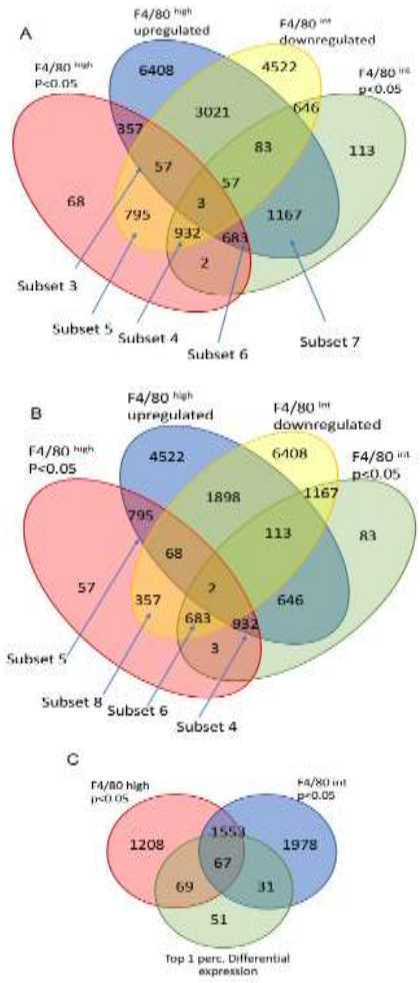


Figure 4.4 *S. mansoni* infection induces profound transcriptional alterations to Kupffer cells and perivascular macrophages

(A, B) The Boolean strategies described in the methods are illustrated in the Venn diagrams: The following set designations were used in the set notation:

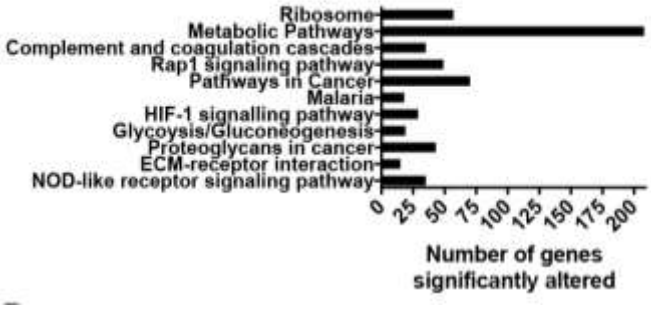
- q_hi = Significantly regulated genes in F4/80high cells (BH adjusted $p<0.05$)
- q_nt = Significantly regulated genes in F4/80int cells (BH adjusted $p<0.05$)
- hi_up = Genes upregulated in F4/80high cells
- int_up = Genes upregulated genes in F4/80int cells
- hi_down = Genes downregulated in F4/80high cells
- int_down = Genes downregulated in F4/80int cells

The employed set notation:

- $A \cap B$ – A intersect B
- $A \cup B$ – A union B
- $A \setminus B$ – A minus B, or A complement B

(C) Top 1-perc. differential expression (normalized log2-foldchange) in perivascular and Kupffer cells

A



Pathway	p-value
Ribosome	2.98E-20
Metabolic Pathways	1.04E-08
Complement and coagulation cascades	3.26E-06
Rap1 signaling pathway	5.11E-05
Pathways in Cancer	2.00E-03
Malaria	3.00E-03
HIF-1 signalling pathway	4.00E-03
Glycoysis/Gluconeogenesis	6.00E-03
Proteoglycans in cancer	0.01
ECM-receptor interaction	0.039
NOD-like receptor signaling pathway	0.041
Fructose and mannose metabolism	0.044

B

Perivascular Macrophages

Pathway	p-value
Ribosome	2.98E-20
Metabolic Pathways	1.04E-08
Complement and coagulation cascades	3.26E-06
Rap1 signaling pathway	5.11E-05
Pathways in cancer	2.00E-03
Malaria	3.00E-03
HIF-1 signalling pathway	4.00E-03
Glycoysis/Gluconeogenesis	0.006
Proteoglycans in cancer	0.01
ECM-receptor interaction	0.039
NOD-like receptor signaling pathway	0.041
Fructose and mannose metabolism	0.044

C

Kupffer cells

Pathway	p-value
Ribosome	1.56E-18
Metabolic Pathways	2.00E-13
Complement and coagulation cascades	3.33E-06
Drug metabolism-cytochrome P450	2.00E-05
Chemokine signaling pathway	2.88E-05
Rap1 signaling pathway	1.91E-04
Lysosome	2.34E-04
Pathways in cancer	2.00E-03
Osteoclast differentiation	2.00E-03
Systemic lupus erythematosus	2.00E-03
Glutathione metabolism	2.00E-03
Cytokine-cytokine receptor interaction	3.00E-03
HIF-1 signalling pathway	3.00E-03
NOD-like receptor signaling pathway	4.00E-03
Proteoglycans in cancer	5.00E-03
Biosynthesis of amino acids	6.00E-03
Glycoysis/Gluconeogenesis	3.00E-03
Glucagon signaling pathway	0.02
PPAR signaling pathway	0.02

Figure 4.5 *S. mansoni* infection induces profound alterations to metabolic pathways in hepatic macrophages.

(A) Pathways that are significantly altered in both perivascular macrophages and Kupffer cells at 10-weeks post *S. mansoni* infection (B) Pathways altered in perivascular macrophages (C) Altered pathways in Kupffer cells.

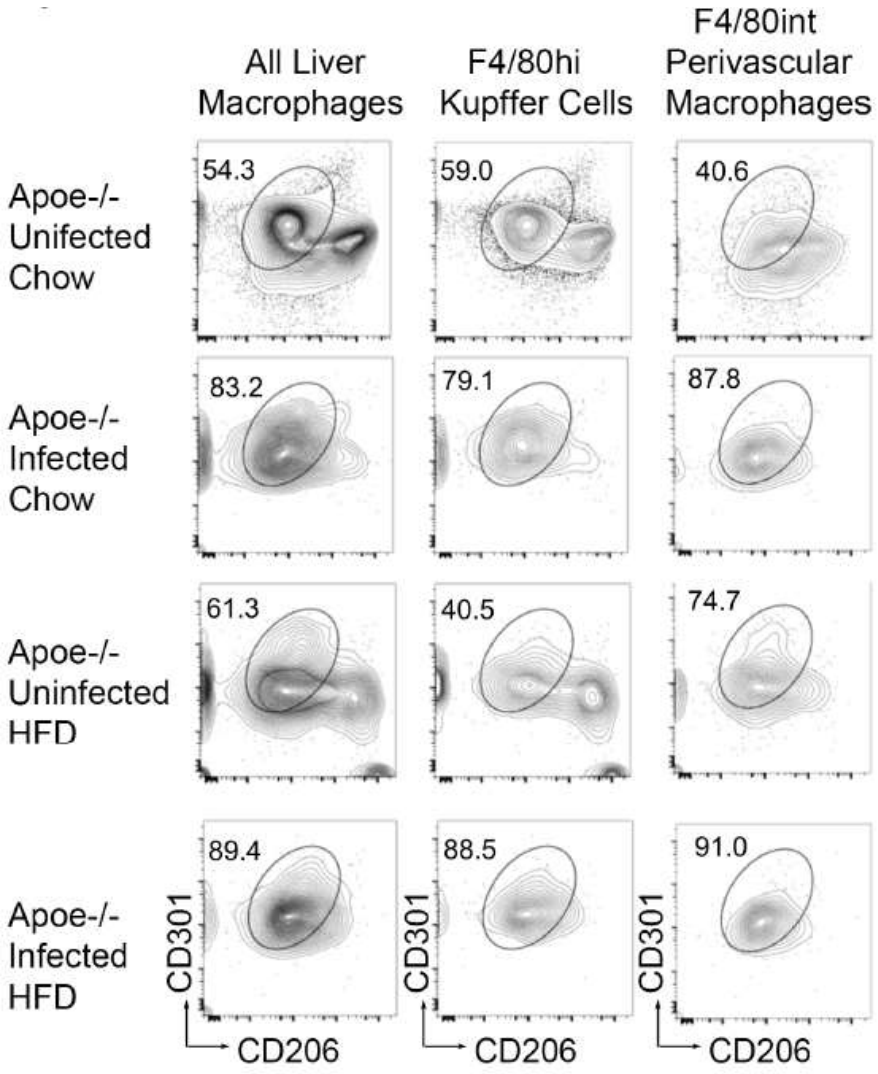


Figure 4.6 *S. mansoni* infection induces alternative macrophage activation even in the presence of high fat diet.

At 10-weeks post infection, mice were sacrificed. Single cell suspension from perfused liver were stained with markers of interest and analyzed by flow cytometry using the macrophage gating strategy depicted in Figure 4.1. Data are concatenated from 5-9 mice per group and representative of four independent experiments

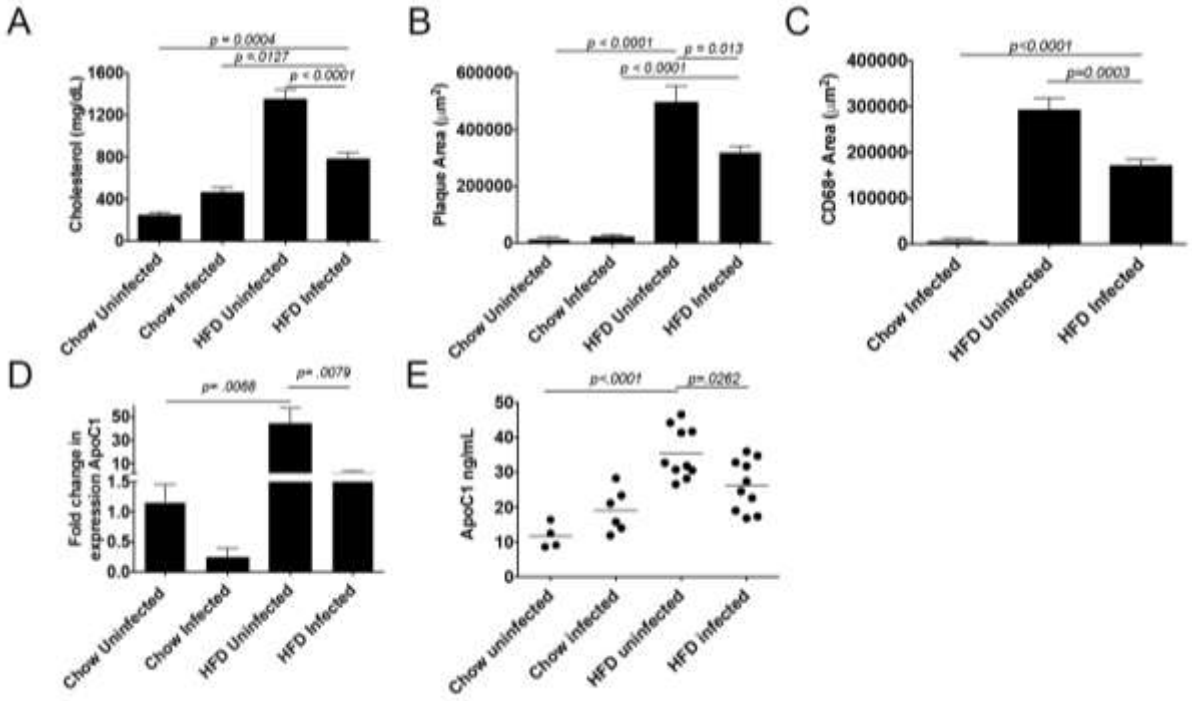


Figure 4.7 *S. mansoni* infection reduces hyperlipidemia, plaque, and macrophage area and well as systemic ApoC1 production in HFD fed ApoE-/- mice. ApoE-/- male mice were fed HFD or standard chow diet for 10 days before infection with S. mansoni.

At 10-weeks post infection, mice were sacrificed. (A) Fasting plasma cholesterol measurements. (B-C) Quantitation of plaque and macrophage area (CD68+) from the aortic sinus. (D) QPCR analysis of ApoC1 in whole liver RNA, data are normalized to Chow infected animals. (E) Quantitation of ApoC1 in plasma at the time of sacrifice. (F) QPCR analysis of CD36 in whole liver RNA, data are normalized to Chow infected animals. All data shown are two combined experiments with 4-8 mice per group in each experiment. The experiment was performed 4 times. Statistical significance was calculated using ANOVA and post-tests.

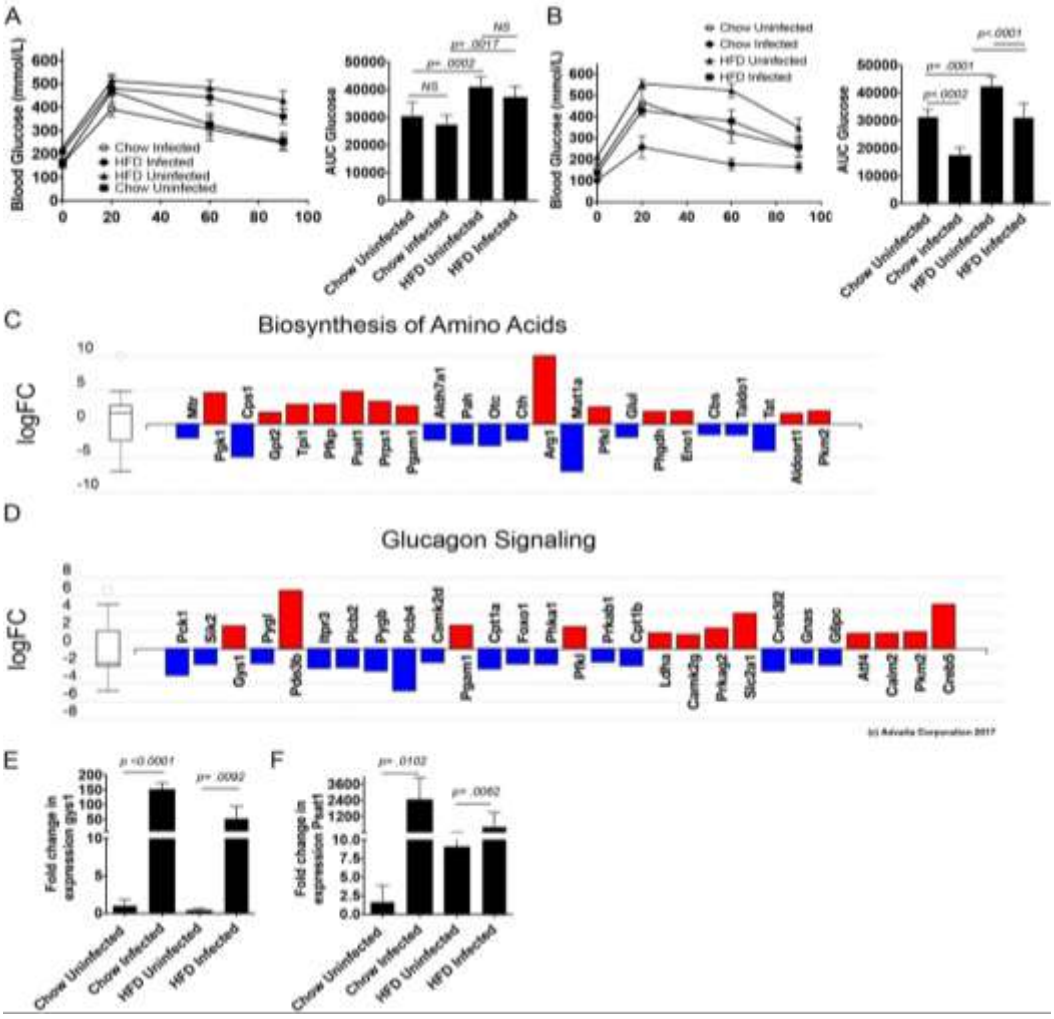


Figure 4.8 *S. mansoni* infection improves glucose tolerance and alters hepatic metabolism towards glycogen storage and amino acid biosynthesis.

(A) and 10- (B) weeks post infection mice were fasted for 5 hours and then administered an i.v GTT. (C-D) Perivascular macrophages and Kupffer cells were sorted from naïve and 10-week S. mansoni-infected C57BL/6 mice, RNA was extracted following Immgen protocols and populations were analyzed using whole-mouse genome Affymetrix Mouse Gene 2.0 ST Arrays. Significantly up (red) and down (blue) -regulated pathways were identified using Advaita Corporation iPathwayGuide. (E-F) QPCR analysis of gys1 and psat1 in whole liver RNA, data are normalized to Chow infected animals. Data in A, B, E, and F are two combined experiments with 4-8 mice per group in each experiment and the experiment was performed 4 times. Statistical significance was calculated using ANOVA and post-test.

CHAPTER 5. SUMMARIZING DISCUSSION

Unexplored role of Interleukins in the modulation of lymphoid organization

In the past few years, a large body of data regarding the multiple functions of interleukins, and in specific of interleukin-4, have emerged. Elegant experiments have revealed the diverse functions of IL-4 in immunity, especially in type 2 responses and more recently, in stromal cells. Lee and coworkers identified a role of IL-4/IL-13 signaling in postnatal expansion of PDGFRα positive mesenchymal cells, as well as in dictating the expansion of adipocytes precursors (Lee et al., 2015). However, despite a growing body of literature pointing to the requirement of IL-4 for hematopoietic and non-hematopoietic cell expansion (as discussed in **Chapter 2**) the role of IL-4 in lymphoid organs, and in lymphoid organization per se have not fully been explored. IL-4 in secondary lymphoid organs had previously been recognized to support a type 2 B cell response, and in a model of antigenic challenge with a helminth (*H. polygyrus*), T follicular helper cells appeared to be the main producers of IL-4 in the lymph node (King & Mohrs, 2009). Additionally, using an *in vitro* lymphatic endothelial cell culture Shin et al. showed that recombinant IL-4 (and IL-13) inhibited lymphatic vessels density and function, and in a model of allergic asthma, IL-4 blockade resulted in increased trafficking of antigen (red fluorescent protein- labelled polystyrene beads) into mediastinal lymph node (Shin et al., 2015). In comparison to these models we also have observed a need for IL-4 in stromal cell expansion in the peripheral lymph node (**Chapter 2**) that depends on the expression of IL-4Rα in these cells. We, as well as other studies (Lee et al., 2015; Yamada, Yamakawa, Imai, & Tsukamoto, 1997), have confirmed the expression of IL-4Rα in FDC, LEC and FRC. Yet, in contrast to Shin et al, who observed a negative effect of IL-4 in lymphoid vessels, we observed that IL-4 is required to maintain the numbers of lymphatic

endothelial cells in the lymph node, both during homeostasis and following challenge with type 1 or type 2 antigens. One possible explanation to this discrepancy is that our studies are based on *ex vivo* analysis on LNs of IL-4Rα deficient mice at steady state, whereas Shin and coworkers did not explore the effects of IL-4 neutralization during homeostasis in the lymph node, but in a Matrigel-based cell culture with a single dose of neutralizing antibody. Still, in our model the effects of IL-4 on FDC, FRC and LEC function and expression of activation markers has not been explored, and it will be the focus of future studies.

Importantly, in **Chapter 2**, we described a marked disorganization of the stromal and lymphocytic compartment, which is of special relevance because many immune cells rely on their localization and cross-talk with neighboring cells to exert their functions, and in some instances, these factors dictate their response to therapies (Halama et al., 2009; Valeyev et al., 2010). Nonetheless, questions still remain, such as what are the specific requirements of IL-4 in each of these stromal cell populations and how cell specific deficiency of IL-4 impacts the response to antigens. As part of these future directions, we plan to knock out IL-4Rα in PDGFRβ-cre cells to know how the lack of IL-4/IL-13 pathway affects lymphoid compartmentalization and cellular response to antigens. PDGFRβ+ cells have been previously identified as precursors of follicular dendritic cells (citation). A caveat to this approach that is worth mentioning, though is that PDGFRβ has also been described as marker for adipocyte precursors (Lee et al., 2015) as well as in a pericyte like population, also identified as double negative cells in lymphoid organs (D. Malhotra et al., 2012). This caveat reveals that knowledge of lineage, function, localization as well heterogeneity of cells of mesenchymal origin remain unclear in the field (Novkovic, Onder, Cheng, Bocharov, & Ludewig, 2018), which highlights the importance of this study to provide insights into the mechanistic requirements of secondary lymphoid organs and the cells that compose them.

Critical signaling pathways in the maintenance of lymph node

Several signaling pathways have been identified in the development and maintenance of lymphoid organs. In lymph nodes, CLEC-2, a receptor for podoplanin, is essential for late stages of LN anlage following birth (Benezech et al., 2014). Similarly, RANKL (Receptor activator of nuclear factor kappa-B ligand, also called TRANCE, OPGL or ODF) is required for the development of peripheral lymph nodes (Dougall et al., 1999; Hess et al., 2012). BAFF (B lymphocyte activating factor of the tumor necrosis factor family) is necessary for naïve B cell survival in the marginal zone, dendritic cell maturation ,and formation of a follicular dendritic cell matrix (Moisini & Davidson, 2009). Additionally, lymphotoxin α and lymphotoxin β, members of the Tumor Necrosis Factor (TNF) family are essential for lymph node genesis (McCarthy et al., 2006; Rennert, James, Mackay, Browning, & Hochman, 1998). Previously, Lundell et al observed that type I and type II Interferon can induce BAFF expression in decidual stromal cells (Lundell et al., 2017). In **Chapter 2** we described a role of IL-4 in the induction of expression of lymphotoxin alpha and beta, both at the transcriptional and translational level. Although further experiments to clarify if this is a direct or indirect regulatory role of IL-4. Moreover, the source of lymphotoxin in these experiments is unknown and will be the focus of future directions (although previous work demonstrated that B and T cells are sources in the lymph node). Furthermore, it is not clear at which point in gestational development the requirement for IL-4 initiates. To explore this, it would necessary to treat mice with recombinant IL-4 during gestation at different timepoints to determine if levels of lymphotoxin are recovered. As the mesenteric lymph node has been shown to be the first to develop in at around 10.5-15.5 gestational days (Spahn & Kucharzik, 2004) it would be necessary to treat mice early during embryogenesis.

Suppression of immune response in maternal infection models

Studies of the effects of prenatal exposure to malaria antigens in offspring have revealed that sensitization in utero leads to a state of immunological anergy that is dependent on IL-10 and Tregs (I. Malhotra et al., 2009). In addition, vaccine failure has been recognized in various prospective studies of children that were antenatally exposed to parasitic antigens (Labeaud, Malhotra, King, King, & King, 2009; D. Malhotra et al., 2012; Ondigo et al., 2018). Additional studies have shown that in helminth infected mothers, offspring (newborn's cord) have higher levels of IL-8, and prenatal exposure also correlated to higher IL-6 and TNF alpha (Olateru-Olagbegi, Omoruyi, Dada, Edem, & Arinola, 2018). In **Chapter 3** we determined that offspring from *S. mansoni* infected mothers exhibited reduced levels of IL-4 at steady state and following immunization with an aluminum adjuvanted tetanus diphtheria vaccine. This reduction correlated with reduced cellular response of plasma cells and follicular dendritic cells, which are essential for antigen persistence and maintenance of B cells through its complement receptors CR1 (CD35) and CR2 (CD21). Moreover, in our model, at steady state the source of IL-4 is invariant NK T cells, which have been previously associated with production of IL-17 in peripheral lymph node, and a smaller fraction of NK1.1^{-} iNKT were capable of producing IL-4 (Doisne et al., 2009). Further characterization of the LN to determine the signals that are required in offspring to drive adequate follicular dendritic cells expansion and the possible functional defects of these population would expand our understanding of the effects that maternal infection confers on offspring. One possible approach to tackle this question is to perform RNAseq analysis in sorted FDC from multiple pups from infected and uninfected mothers. This will provide insights into the regulatory/differentiation pathways that are affected following antenatal exposure to parasites.

Memory response in mice and humans

One of the ultimate goals with vaccination is the induction of immunological memory, which is defined as the ability of the immune system to mount rapid responses upon re-exposure with the same antigen (Bergmann et al., 2013). Evidence that memory B cells arise from GC reactions come from studies in mice and humans where weak GC translated into impaired somatic mutation in memory B cells and recall responses (Bergmann et al., 2013; Good-Jacobson & Shlomchik, 2010; Shlomchik & Weisel, 2012). In comparison to these studies, in **Chapter 3,** we show diminished GC formation in pups from infected mother in comparison to the control group (uninfected pups) which correlates to decreased memory B cell response, both at 8 days and during recall response at 60 days post immunization. It is still unclear what signals determine the quality, length, and quantity of the memory response and plasma cell formation in our model. We hypothesize in **Chapter 3** that some of these defects stem from a deficiency in both total numbers of FDCs, which are required for the formation of an antigen-antibody C3 complex to generate memory B cells(Klaus & Humphrey, 1977), and a reduction in CD21/35 expression on these cells (Haas & Tedder, 2005). Added to this, IL-4 receptor is required to develop protective memory T cells in a murine model of malaria infection (Morrot, Hafalla, Cockburn, Carvalho, & Zavala, 2005). Importantly, crosstalk of Tfh and B cells is necessary to sustain GC reactions, as IL-21 derived Tfh induces plasma cells differentiation (Zhang et al., 2018). Furthermore, the rise of Tfh following a secondary stimulus is dependent on long lasting memory cells and is dependent on the recruitment of IL-7R+ cells T cells. The enhanced plasmablast development also requires recruitment from the memory T cells (Fairfax et al., 2015). Additionally, IL-4 in a secondary response is dependent on memory B cells and naïve B cells, but not GC B cells, as depletion of these populations (by anti-CD20 mAb) led to a reduced Tfh expansion and IL-4 levels in the

lymph node (Fairfax et al., 2015). In our study we observed decreased IL-4 levels that correlate to decreased memory T cells, therefore it is possible a combination of IL-4 deficiency and reduced follicular dendritic cells cause reduced memory cell populations. More recently, the role of epigenetic modifications in the regulation of memory responses have been recognized. Expression of memory markers such as CD38 and CD27 are a consequence of *de novo* activating histone modifications (Wu et al., 2018). DNA methylation inhibits plasma cells differentiation and B cell activation and differentiation into plasma cells (Barwick et al., 2018). Parasitic antigens and maternal antibodies have been shown to be passively transmitted during breastfeeding (Hanson, 1998; Jackson & Nazar, 2006; Santos et al., 2016); in addition, breastfeeding affects DNA methylation in the offspring (Hartwig, Loret de Mola, Davies, Victora, & Relton, 2017). Long term future studies should strive to determine the possible epigenetics modifications in offspring that were exposed to parasitic antigens antenatally.

Implications of athero-protective Schistosoma-mediated reprograming of macrophages

In **Chapter 4** we confirmed the anti-atherogenic effect of schistosomiasis and suggested a putative mechanism by which Schistosomiasis exerts protection against atherosclerosis. Previously, Doenhoff et al. reported that mice infected with *S. mansoni* presented lower total cholesterol (Doenhoff, Stanley, Griffiths, & Jackson, 2002). Similarly, Da Fonseca reported lower plasma lipids in patients with hepatosplenic schistosomiasis (Martins da Fonseca et al., 2014). Moreover, Stanley et al. reported that while *S. mansoni* eggs had a lowering effect on plasma cholesterol, adult *S. mansoni* worms had no effect (Stanley, Jackson, Griffiths, & Doenhoff, 2009). In **Chapter 4** we propose that Schistosomiasis athero-protection is in part, due to metabolic reprogramming of hepatic macrophages, since the infection causes profound alterations in the expression of genes within the phospholipid and cholesterol pathways. Furthermore, such effects

are mediated at least in part by downregulation of apoA1, apoB, and apoC1. Previously, Westertep et al showed that overexpression of apoC1 correlates with hypercholesterolemia and atherosclerosis development (Westerterp et al., 2007). We confirmed that in our model, total serum ApoC1 was significantly reduced and postulated that athero-protection was in part due to reductions in serum ApoC1. Although experiments will need to be performed to unequivocally demonstrate the contribution of macrophage-derived apoC1 to the development of atherosclerosis in apoE$^{-/-}$ mice. One of the possible approaches for this is to ablate macrophages by using dichloromethylene bisphosphonate (clodronate liposome) in the liver of infected mice following by cell transfer of monocytes from apoC1$^{-/-}$ and APOC1 transgenic (overexpressing apoC1) and determine plasma cholesterol and aortic plaque lesions 14 weeks post cell transfer to determine the role of ApoC1 derived in atherosclerosis. If reduced plaque and cholesterol are observed in mice that were transferred cells from apoC1$^{-/-}$ we can then conclude that ApoC1 by itself is sufficient to protect from atherosclerosis.

Immunometabolism of Macrophages

Historically, macrophages' role in atherosclerosis have been recognized principally due to their ability to ingest lipoprotein , leading to the formation of foam cells and subsequently to plaque instability and rupture (Zeller & Srivastava, 2014). However, emerging evidence points to a beneficial role of macrophages in atherosclerosis and obesity-induced insulin resistance (Fitzgibbons & Czech, 2016). Nevertheless, most of this evidence is based on the capacity of macrophages to undergo autophagy, thus reducing extracellular particle matter such as cholesterol crystals that serve as stimulus for inflammasome activation (Razani et al., 2012). The M2 (alternatively activated) macrophages and their expression markers have been associated with reduced plaque vulnerability and ischemia (Cho et al., 2013), while M1 macrophages are

associated with increased inflammation in atherosclerotic plaques. Although archetypal markers associated to macrophage classical or alternative activation have been widely described within macrophages and their relationship to atherosclerosis, the variation of their intracellular metabolism and how they regulate the functional abilities of these cells is emerging and becoming a topic of great interest and discussion (Groh, Keating, Joosten, Netea, & Riksen, 2018). Combined transcriptional and metabolic network analysis showed that typically, M1 macrophages synthesize nitric oxide (NO) from L-arginine via inducible-nitric oxide synthase, producing NO and aiding in processes like angiogenesis. Also, M1 macrophages use glucose primarily via glycolysis and possess a truncated tricarboxylic acid cycle (TCA) which leads to accumulation of succinate, that results in HIF-alpha stabilization and pro-inflammatory gene transcription (Geeraerts, Bolli, Fendt, & Van Ginderachter, 2017). On the other hand, M2 macrophages are characterized by energy production (ATP synthesis) by oxidative TCA cycle, oxidative phosphorylation (OXPHOS) and fatty acid oxidation (Huang et al., 2014; Vats et al., 2006), as well as arginine catabolism via arginase (Arg1) that produces L-ornithine and L-proline, which is essential for collagen production and wound healing and tissue repair (Kelly & O'Neill, 2015).

In **Chapter 4**, we described two subsets of liver macrophages (resident, Kupffer macrophages and perivascular macrophages) that exhibit M2 markers and possess a unique metabolic phenotype following infection with the helminth *Schistosoma mansoni*, with over 200 genes involved in metabolism significantly altered. The reprogramming of hepatic macrophages following infection was characterized by enhanced expression of genes such as Atp6v0d2, involved in insulin receptor recycling; asns, which catalyzes the synthesis of asparagine and glutamate from aspartate and glutamine in an ATP-dependent amidotransferase reaction (Lomelino, Andring, McKenna, & Kilberg, 2017) and gda, member of the purine metabolism

pathway. While plcb4, a phospholipase was downregulated. Combining transcriptomics with metabolomics analysis will help elucidate critical pathways that help in the regulation of macrophage metabolism and atherosclerosis protection. Moreover, future directions should strive to further expand the emerging role of microRNAs in the regulation of macrophage metabolism. MicroRNAs such as miR-33 controls lipid metabolism and its disruption induced macrophage polarization to a M2 phenotype, thus regulating adaptive immune responses. (Ouimet et al., 2015). miR-155 has raised as both a pro-inflammatory and anti-inflammatory regulator, with multiple studies suggesting distinct roles for this microRNA (Martinez-Nunez, Louafi, & Sanchez-Elsner, 2011; Tili et al., 2007). These opposing results reveals the major challenges in understanding the role of microRNA in macrophage immunometabolism, which is something we will strive to comprehend in future studies.

Working Models: Conclusions

Model 1. IL-4 regulation of stromal-lymphocyte cell homeostasis in the lymph node

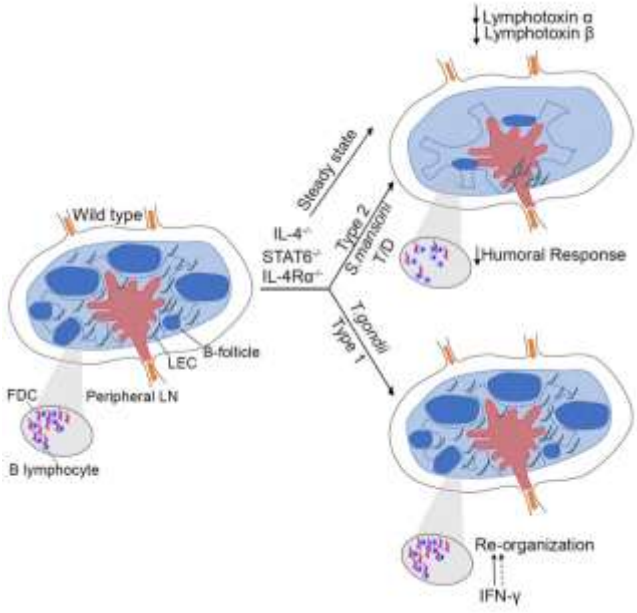


Figure 5.1. Summarizing schematics of chapter 2

Besides its effect in Th2 responses, IL-4 is also necessary to sustain the stromal-lymphocyte axis in peripheral lymph nodes at steady state. Deficiency of IL-4/IL4Rα pathway lead to reduced numbers of follicular dendritic cells and lymphatic endothelial cells. In addition, this reduction correlated to disorganized lymph nodes at steady state. IL-4 deficiency correlated to reduced lymphotoxin alpha and beta, but not to

reductions in BAFF. Although, it is not clear how IL-4 downregulates lymphotoxin expression, it was clear that lymphotoxin expression was rescued by a Type 1inducing immunization, which was in part mediated by IFN-γ, but not by induction of a Type 2 response. Importantly, defects in cellularity of FDCs and LECs following a Type 2 (Tetanus-diphtheria) immunization correlated to a diminished humoral response against diphtheria, but not against Tetanus. Overall, this study revealed a fundamental role in the IL-4 pathway for maintenance of vital stromal cell populations in peripheral lymph node and its role in sustaining an appropriate immune response against specific antigens.

Model 2: Maternal Schistosomiasis: Effects on offspring immunity

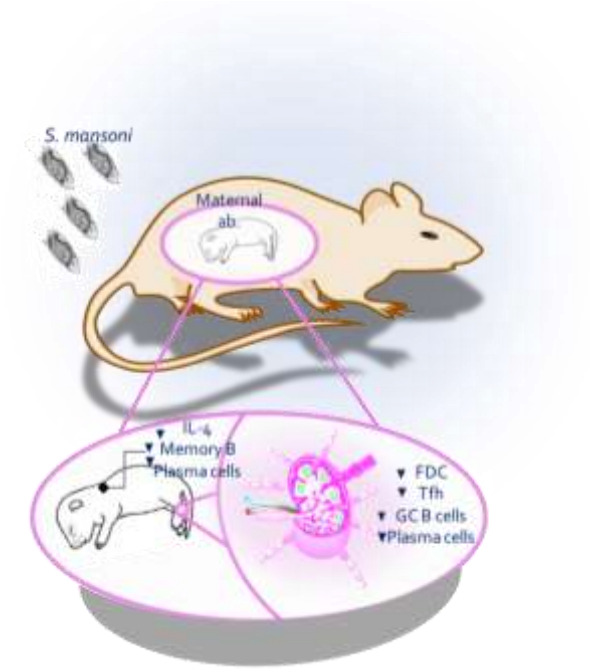


Figure 5.2. Summarizing schematic of chapter

Schistosomiasis during pregnancy is responsible for stillbirths and low birth weight. In addition, a majority of human and mouse studies suggest a disadvantageous effect of maternal infection on the offspring's response to vaccination. Although the mechanism(s) that lead to a state of immune hypo-responsiveness in the offspring from infected mothers are still not completely understood, our study suggests that this is in part, due to reduced IL-4 production in offspring from infected mothers in comparison to controls from uninfected mothers. Reduction in IL-4 correlates to reduced plasma cells at steady state. Moreover, reduction of IL-4 correlated to reduced follicular dendritic cells, known to be

necessary for antigen persistence and complement fixed-antigen presentation to B cells. Reduction in this critical population in lymph nodes lead to diminished germinal centers and plasma cell expansion following immunization. These novels results help us understand and propose better immunization regimens, especially in regions where the disease is endemic.

Model 3: Schistosomiasis induced protection from atherosclerosis: Contribution of IL-4 vs. SEA

Schistosomiasis induces metabolic reprogramming of hepatic macrophages. The relative contributions of IL-4 and SEA are not well understood. The reprogramming of macrophages upregulated genes such as Arg1 and nos 2(M2 phenotype archetypal markers), and it induced the upregulation of genes involved in metabolic pathways which included ApoC1, Psat and Gys1.

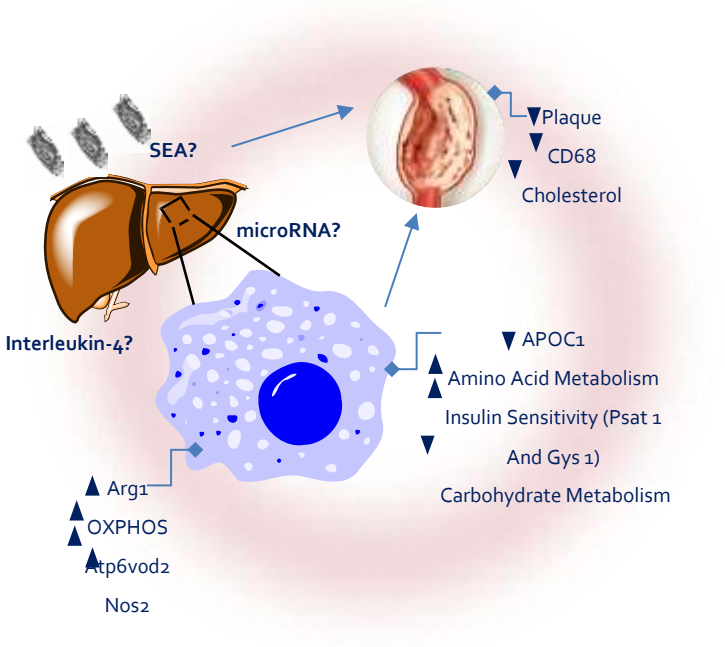


Figure 5.3. Summarizing schematic of chapter 4

Metabolic pathway upregulation in hepatic macrophages correlated to decreased cholesterol and plaque formation in a mouse model of atherosclerosis. Overall, this study suggests a possible mechanism by which Schistosomiasis protects against atherosclerosis and potentially reveals possible pathways that can be exploited in the development of treatments against the disease.

References

Barwick, B. G., Scharer, C. D., Martinez, R. J., Price, M. J., Wein, A. N., Haines, R. R., . . . Boss, J. M. (2018). B cell activation and plasma cell differentiation are inhibited by de novo DNA methylation. *Nat Commun, 9*(1), 1900. doi:10.1038/s41467-018-04234-4

Benezech, C., Nayar, S., Finney, B. A., Withers, D. R., Lowe, K., Desanti, G. E., . . . Barone, F. (2014). CLEC-2 is required for development and maintenance of lymph nodes. *Blood, 123*(20), 3200-3207. doi:10.1182/blood-2013-03-489286

Bergmann, B., Grimsholm, O., Thorarinsdottir, K., Ren, W., Jirholt, P., Gjertsson, I., & Martensson, I. L. (2013). Memory B cells in mouse models. *Scand J Immunol, 78*(2), 149-156. doi:10.1111/sji.12073

Cho, K. Y., Miyoshi, H., Kuroda, S., Yasuda, H., Kamiyama, K., Nakagawara, J., . . . Atsumi, T. (2013). The phenotype of infiltrating macrophages influences arteriosclerotic plaque vulnerability in the carotid artery. *J Stroke Cerebrovasc Dis, 22*(7), 910-918. doi:10.1016/j.jstrokecerebrovasdis.2012.11.020

Doenhoff, M. J., Stanley, R. G., Griffiths, K., & Jackson, C. L. (2002). An anti-atherogenic effect of Schistosoma mansoni infections in mice associated with a parasite-induced lowering of blood total cholesterol. *Parasitology, 125*(Pt 5), 415-421.

Doisne, J. M., Becourt, C., Amniai, L., Duarte, N., Le Luduec, J. B., Eberl, G., & Benlagha, K. (2009). Skin and peripheral lymph node invariant NKT cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions. *J Immunol, 183*(3), 2142-2149. doi:10.4049/jimmunol.0901059

Dougall, W. C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., . . . Schuh, J. (1999). RANK is essential for osteoclast and lymph node development. *Genes Dev, 13*(18), 2412-2424.

Fairfax, K. C., Everts, B., Amiel, E., Smith, A. M., Schramm, G., Haas, H., . . . Pearce, E. J. (2015). IL-4-secreting secondary T follicular helper (Tfh) cells arise from memory T cells, not persisting Tfh cells, through a B cell-dependent mechanism. *J Immunol, 194*(7), 2999-3010. doi:10.4049/jimmunol.1401225

Fitzgibbons, T. P., & Czech, M. P. (2016). Emerging evidence for beneficial macrophage functions in atherosclerosis and obesity-induced insulin resistance. *J Mol Med (Berl), 94*(3), 267-275. doi:10.1007/s00109-016-1385-4

Geeraerts, X., Bolli, E., Fendt, S. M., & Van Ginderachter, J. A. (2017). Macrophage Metabolism As Therapeutic Target for Cancer, Atherosclerosis, and Obesity. *Front Immunol, 8*, 289. doi:10.3389/fimmu.2017.00289

Good-Jacobson, K. L., & Shlomchik, M. J. (2010). Plasticity and heterogeneity in the generation of memory B cells and long-lived plasma cells: the influence of germinal center interactions and dynamics. *J Immunol, 185*(6), 3117-3125. doi:10.4049/jimmunol.1001155

Groh, L., Keating, S. T., Joosten, L. A. B., Netea, M. G., & Riksen, N. P. (2018). Monocyte and macrophage immunometabolism in atherosclerosis. *Semin Immunopathol, 40*(2), 203-214. doi:10.1007/s00281-017-0656-7

Haas, K. M., & Tedder, T. F. (2005). Role of the CD19 and CD21/35 receptor complex in innate immunity, host defense and autoimmunity. *Adv Exp Med Biol, 560*, 125-139. doi:10.1007/0-387-24180-9_16

Halama, N., Michel, S., Kloor, M., Zoernig, I., Pommerencke, T., von Knebel Doeberitz, M., . . . Jager, D. (2009). The localization and density of immune cells in primary tumors of human metastatic colorectal cancer shows an association with response to chemotherapy. *Cancer Immun, 9*, 1.

Hanson, L. A. (1998). Breastfeeding provides passive and likely long-lasting active immunity. *Ann Allergy Asthma Immunol, 81*(6), 523-533; quiz 533-524, 537. doi:10.1016/S1081-1206(10)62704-4

Hartwig, F. P., Loret de Mola, C., Davies, N. M., Victora, C. G., & Relton, C. L. (2017). Breastfeeding effects on DNA methylation in the offspring: A systematic literature review. *PLoS One, 12*(3), e0173070. doi:10.1371/journal.pone.0173070

Hess, E., Duheron, V., Decossas, M., Lezot, F., Berdal, A., Chea, S., . . . Mueller, C. G. (2012). RANKL induces organized lymph node growth by stromal cell proliferation. *J Immunol, 188*(3), 1245-1254. doi:10.4049/jimmunol.1101513

Huang, S. C., Everts, B., Ivanova, Y., O'Sullivan, D., Nascimento, M., Smith, A. M., . . . Pearce, E. J. (2014). Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat Immunol, 15*(9), 846-855. doi:10.1038/ni.2956

Jackson, K. M., & Nazar, A. M. (2006). Breastfeeding, the immune response, and long-term health. *J Am Osteopath Assoc, 106*(4), 203-207.

Kelly, B., & O'Neill, L. A. (2015). Metabolic reprogramming in macrophages and dendritic cells in innate immunity. *Cell Res, 25*(7), 771-784. doi:10.1038/cr.2015.68

King, I. L., & Mohrs, M. (2009). IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells. *J Exp Med, 206*(5), 1001-1007. doi:10.1084/jem.20090313

Klaus, G. G., & Humphrey, J. H. (1977). The generation of memory cells. I. The role of C3 in the generation of B memory cells. *Immunology, 33*(1), 31-40.

Labeaud, A. D., Malhotra, I., King, M. J., King, C. L., & King, C. H. (2009). Do antenatal parasite infections devalue childhood vaccination? *PLoS Negl Trop Dis, 3*(5), e442. doi:10.1371/journal.pntd.0000442

Lee, M. W., Odegaard, J. I., Mukundan, L., Qiu, Y., Molofsky, A. B., Nussbaum, J. C., . . . Chawla, A. (2015). Activated type 2 innate lymphoid cells regulate beige fat biogenesis. *Cell, 160*(1-2), 74-87. doi:10.1016/j.cell.2014.12.011

Lomelino, C. L., Andring, J. T., McKenna, R., & Kilberg, M. S. (2017). Asparagine synthetase: Function, structure, and role in disease. *J Biol Chem, 292*(49), 19952-19958. doi:10.1074/jbc.R117.819060

Lundell, A. C., Nordstrom, I., Andersson, K., Lundqvist, C., Telemo, E., Nava, S., . . . Rudin, A. (2017). IFN type I and II induce BAFF secretion from human decidual stromal cells. *Sci Rep, 7*, 39904. doi:10.1038/srep39904

Malhotra, D., Fletcher, A. L., Astarita, J., Lukacs-Kornek, V., Tayalia, P., Gonzalez, S. F., . . . Immunological Genome Project, C. (2012). Transcriptional profiling of stroma from inflamed and resting lymph nodes defines immunological hallmarks. *Nat Immunol, 13*(5), 499-510. doi:10.1038/ni.2262

Malhotra, I., Dent, A., Mungai, P., Wamachi, A., Ouma, J. H., Narum, D. L., . . . King, C. L. (2009). Can prenatal malaria exposure produce an immune tolerant phenotype? A prospective birth cohort study in Kenya. *PLoS Med, 6*(7), e1000116. doi:10.1371/journal.pmed.1000116

Martinez-Nunez, R. T., Louafi, F., & Sanchez-Elsner, T. (2011). The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J Biol Chem, 286*(3), 1786-1794. doi:10.1074/jbc.M110.169367

Martins da Fonseca, C. S., Pimenta Filho, A. A., dos Santos, B. S., da Silva, C. A., Domingues, A. L., Owen, J. S., & Lima, V. L. (2014). Human plasma lipid modulation in schistosomiasis mansoni depends on apolipoprotein E polymorphism. *PLoS One, 9*(7), e101964. doi:10.1371/journal.pone.0101964

McCarthy, D. D., Summers-Deluca, L., Vu, F., Chiu, S., Gao, Y., & Gommerman, J. L. (2006). The lymphotoxin pathway: beyond lymph node development. *Immunol Res, 35*(1-2), 41-54. doi:10.1385/IR:35:1:41

Moisini, I., & Davidson, A. (2009). BAFF: a local and systemic target in autoimmune diseases. *Clin Exp Immunol, 158*(2), 155-163. doi:10.1111/j.1365-2249.2009.04007.x

Morrot, A., Hafalla, J. C., Cockburn, I. A., Carvalho, L. H., & Zavala, F. (2005). IL-4 receptor expression on CD8+ T cells is required for the development of protective memory responses against liver stages of malaria parasites. *J Exp Med, 202*(4), 551-560. doi:10.1084/jem.20042463

Novkovic, M., Onder, L., Cheng, H. W., Bocharov, G., & Ludewig, B. (2018). Integrative Computational Modeling of the Lymph Node Stromal Cell Landscape. *Front Immunol, 9*, 2428. doi:10.3389/fimmu.2018.02428

Olateru-Olagbegi, O. A., Omoruyi, E. C., Dada, R. A., Edem, V. F., & Arinola, O. G. (2018). Serum Levels of Inflammatory Cytokines in Helminth Infested Pregnant Women and Cord Blood of their Babies in Relation to Pregnancy Outcome. *Niger J Physiol Sci, 33*(1), 51-56.

Ondigo, B. N., Muok, E. M. O., Oguso, J. K., Njenga, S. M., Kanyi, H. M., Ndombi, E. M., . . . Colley, D. G. (2018). Impact of Mothers' Schistosomiasis Status During Gestation on Children's IgG Antibody Responses to Routine Vaccines 2 Years Later and Anti-Schistosome and Anti-Malarial Responses by Neonates in Western Kenya. *Front Immunol, 9*, 1402. doi:10.3389/fimmu.2018.01402

Ouimet, M., Ediriweera, H. N., Gundra, U. M., Sheedy, F. J., Ramkhelawon, B., Hutchison, S. B., . . . Moore, K. J. (2015). MicroRNA-33-dependent regulation of macrophage metabolism directs immune cell polarization in atherosclerosis. *J Clin Invest, 125*(12), 4334-4348. doi:10.1172/JCI81676

Razani, B., Feng, C., Coleman, T., Emanuel, R., Wen, H., Hwang, S., . . . Semenkovich, C. F. (2012). Autophagy links inflammasomes to atherosclerotic progression. *Cell Metab, 15*(4), 534-544. doi:10.1016/j.cmet.2012.02.011

Rennert, P. D., James, D., Mackay, F., Browning, J. L., & Hochman, P. S. (1998). Lymph node genesis is induced by signaling through the lymphotoxin beta receptor. *Immunity, 9*(1), 71-79.

Santos, P., Lorena, V. M., Fernandes Ede, S., Sales, I. R., Nascimento, W. R., Gomes Yde, M., . . . Souza, V. M. (2016). Gestation and breastfeeding in schistosomotic mothers differently modulate the immune response of adult offspring to postnatal Schistosoma mansoni infection. *Mem Inst Oswaldo Cruz, 111*(2), 83-92. doi:10.1590/0074-02760150293

Shin, K., Kataru, R. P., Park, H. J., Kwon, B. I., Kim, T. W., Hong, Y. K., & Lee, S. H. (2015). TH2 cells and their cytokines regulate formation and function of lymphatic vessels. *Nat Commun, 6*, 6196. doi:10.1038/ncomms7196

Shlomchik, M. J., & Weisel, F. (2012). Germinal center selection and the development of memory B and plasma cells. *Immunol Rev, 247*(1), 52-63. doi:10.1111/j.1600-065X.2012.01124.x

Spahn, T. W., & Kucharzik, T. (2004). Modulating the intestinal immune system: the role of lymphotoxin and GALT organs. *Gut, 53*(3), 456-465.

Stanley, R. G., Jackson, C. L., Griffiths, K., & Doenhoff, M. J. (2009). Effects of Schistosoma mansoni worms and eggs on circulating cholesterol and liver lipids in mice. *Atherosclerosis, 207*(1), 131-138. doi:10.1016/j.atherosclerosis.2009.04.037

Tili, E., Michaille, J. J., Cimino, A., Costinean, S., Dumitru, C. D., Adair, B., . . . Croce, C. M. (2007). Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol, 179*(8), 5082-5089.

Valeyev, N. V., Hundhausen, C., Umezawa, Y., Kotov, N. V., Williams, G., Clop, A., . . . Nestle, F. O. (2010). A systems model for immune cell interactions unravels the mechanism of inflammation in human skin. *PLoS Comput Biol, 6*(12), e1001024. doi:10.1371/journal.pcbi.1001024

Vats, D., Mukundan, L., Odegaard, J. I., Zhang, L., Smith, K. L., Morel, C. R., . . . Chawla, A. (2006). Oxidative metabolism and PGC-1beta attenuate macrophage-mediated inflammation. *Cell Metab, 4*(1), 13-24. doi:10.1016/j.cmet.2006.05.011

Westerterp, M., Van Eck, M., de Haan, W., Offerman, E. H., Van Berkel, T. J., Havekes, L. M., & Rensen, P. C. (2007). Apolipoprotein CI aggravates atherosclerosis development in ApoE-knockout mice despite mediating cholesterol efflux from macrophages. *Atherosclerosis, 195*(1), e9-16. doi:10.1016/j.atherosclerosis.2007.01.015

Wu, H., Deng, Y., Feng, Y., Long, D., Ma, K., Wang, X., . . . Lu, Q. (2018). Epigenetic regulation in B-cell maturation and its dysregulation in autoimmunity. *Cell Mol Immunol, 15*(7), 676-684. doi:10.1038/cmi.2017.133

Yamada, K., Yamakawa, M., Imai, Y., & Tsukamoto, M. (1997). Expression of cytokine receptors on follicular dendritic cells. *Blood, 90*(12), 4832-4841.

Zeller, I., & Srivastava, S. (2014). Macrophage functions in atherosclerosis. *Circ Res, 115*(12), e83-85. doi:10.1161/CIRCRESAHA.114.305641

Zhang, Y., Tech, L., George, L. A., Acs, A., Durrett, R. E., Hess, H., . . . Toellner, K. M. (2018). Plasma cell output from germinal centers is regulated by signals from Tfh and stromal cells. *J Exp Med, 215*(4), 1227-1243. doi:10.1084/jem.20160832