Induction of T helper 2 cell responses against *Schistosoma mansoni* eggs in the murine intestine

Johannes Urban Mayer
B.Sc., M.Res.

Submitted in fulfilment of the requirement for the degree of
Doctor of Philosophy (PhD)

Institute of Infection, Immunity and Inflammation
College of Medical Veterinary and Life Sciences
University of Glasgow

January 2017

Summary

T helper 2 (Th2) cell responses typify the immune response to parasitic organisms, which frequently invade the intestine. Dendritic cells (DCs) are considered vital for the induction of Th2 responses as they present parasite-derived antigens to naive T cells in draining lymph nodes. However, the identities of the DC populations responsible for priming Th2 cells in the intestine are still unclear. We developed an experimental immunization protocol to deliver *Schistosoma mansoni* eggs into the intestine. During live infection by the parasite, these eggs cause intestinal damage, granuloma formation, tissue fibrosis and strong type 2 immune responses.

Many aspects of type 2 immunity are controlled by the transcription factor IRF4 and we observed that intestinal Th2 responses against *Schistosoma mansoni* eggs did not develop in the draining lymph nodes in the absence of $IRF4^{+}$ DCs. $IRF4^{f/f}$ CD11c-cre positive mice had fewer CD11b-expessing migrating DCs, and fewer parasite antigen-carrying DCs were present in the mesenteric lymph nodes (MLNs) draining the small intestine and colon. However, transfer of antigen-loaded IRF4-deficient DCs directly into the MLN revealed that these cells could induce antigen-specific Th2 responses, suggesting that IRF4 controlled the migration of CD11b-expessing DCs rather than their Th2 inducing capacity.

Furthermore, immature DCs from the intestinal lamina propria, and semi-mature DCs from lymph were sufficient to prime antigen-specific Th2 responses against egg antigens when transferred into naive recipient mice. This induction was dependent on MHCII expression but not on the production of IL-4 by the transferred DCs, indicating that conventional intestinal DCs are fully capable of inducing Th2 responses against *S. mansoni* egg antigens upon transfer.

Further analysis of migratory small intestinal and colonic lymph DCs revealed that distinct subsets of CD11b-expressing DCs were sufficient for the induction of Th2 responses in the small intestine and colon. $CD11b^{+}CD103^{+}$ DCs transported parasite antigen from the small intestine, whereas $CD11b^{+}CD103^{-}$ DCs performed this role in the colon. Of note, these same small intestinal and colonic DC subsets were also the populations that were most efficient at priming antigen-specific Th2 responses *in vivo*.

Thus, we have not only identified that IRF4-dependent CD11b-expressing DCs are specialized to drive Th2 responses in the intestine but have also revealed that different DC subsets promote Th2 responses in the small intestine and colon. These findings not only advance our knowledge of intestinal Th2 responses against parasite antigens but also reveal a hitherto unappreciated functional heterogeneity among intestinal DCs, which could also be relevant for other tissue-specific intestinal conditions like Crohn's disease, ulcerative colitis and celiac disease.

List of Contents

List of Figures

Acknowledgements

First, I would like to thank my PhD supervisor Simon Milling for his help and support over the last three and a half years. He has always had time for me, encouraged and supported my ideas and had helpful suggestions or constructive criticism when I was stuck with experiments or when things seemed clearer in my head than they were on paper.

I would also like to thank the Wellcome Trust PhD program, which has not only provided me with a scholarship and funds to conduct my research, but under the watchful eye of Olwyn Byron and Darren Monckton has made this an invaluable experience that went far beyond my actual research project but broadened my horizons and prepared me for a future in science.

It is an impossible task to appropriately thank everybody that has been important to me during this PhD. This is especially true for all the present and past members of the Milling and Mowat laboratories. All of you have made this a very special time for me; be it by teaching me new techniques, developing new ideas, discussing or commenting on my research, lending a helping hand when things got tough, trading and letting me borrow mice and reagents when I did not have enough or just having an open ear to my problems, enjoying a laugh, an evening in the pub or a day-out together.

I would also like to thank the staff of the CRF and the VRF, who have helped with my many animal experiments and have always been extremely helpful and accommodating. My thanks also go to Diane for all her help in setting up the flow cytometers and sorters during my many experiments.

Finally, I would like to thank my family, especially my parents, who have always supported and encouraged me and have raised me to be the curious, independent, free thinking individual that I am today.

Author Declaration

I declare that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature: ..... Printed name: Johannes Urban Mayer

Chapter 1: Introduction

Parasite infections present a huge global health burden that cause chronic diseases and also have wide socioeconomical effects by infecting livestock. Over 2.5 billion people, which is about a third of the world's population, are infected by parasitic worms such as helminths, which mostly reside within the gastrointestinal tract (Hotez et al., 2008). Most parasite infections cause type 2 immune responses, which include innate responses that try to damage or expel the parasite, and adaptive responses that recruit effector cells, support expulsion and produce antibodies. However, many parasites have evolved to persist within the host for many years, and cause chronic morbidity. One example are schistosomes, which are parasitic worms that chronically infect more than 200 million people worldwide and cause an estimated mortality of 280,000 per year (van der Werf et al., 2003). Most of the pathologic symptoms of schistosomiasis are caused by damaging effects of chronic type 2 immune responses against parasite eggs (Vennervald and Polman, 2009), and the aim of this project is to understand how these immune responses are initiated. In this work we focused on immune responses in the intestine, which is heavily damaged by *Schistosoma mansoni* eggs and is the major site of infection of most soil-transmitted helminths parasites. In order to understand the infection of *S. mansoni* and the immune responses that are caused by the eggs within the definitive human host, the immune system is first introduced. We hereby focus on the immune system of the gastrointestinal tract, as it directly relates to the experiments that follow. These experiments were all performed within the mouse but findings from both human and murine studies are introduced to relate to human pathology.

1.1 The intestinal immune system

1.1.1 Intestinal anatomy and physiology

The different regions of the intestine have distinct anatomical features, which have specialized to support their primary function of nutrient and water uptake. The intestine is constantly exposed to molecules from the outside world and harbours the largest number of immune cells in the body to tolerate and if necessary defend against these foreign antigens. It is anatomically divided into the small intestine and the colon. The small intestine starts after the stomach and consists of the duodenum, the jejunum and the ileum. It is arranged in multiple coils and can reach up to 7 meters of length in humans. The main function of the small intestine is the uptake of nutrients, which is performed by microvillous enterocytes, which line the epithelium, have a large surface area and are specialized for nutrient digestion and uptake. The surface area of the small intestine is further amplified by finger-like projections known as villi, which extend into the intestinal lumen. Epithelial cells, such as enterocytes, mucus-secreting goblet cells and Paneth cells are continuously renewed by multipotent stem cells, which reside in the crypts at the bottom of the villi, and are fully replaced every 4-5 days (Mowat and Agace, 2014). Apart from enterocytes and goblet cells, intraepithelial lymphocytes (IELs) are present within the epithelial layer of the villi, and help maintain epithelial integrity.

Food allergies and celiac disease are restricted to the small intestine as these conditions represent immune responses that are directed against food antigens. In food allergy, pathologic type 2 immune responses are activated by food antigens and innate immune cells mount an acute reaction immediately after allergen exposure, which is amplified by T cells and allergen specific antibodies (Smythies et al., 2005; Johnston et al., 2014). Celiac disease, on the other hand, is a genetic autoimmune disorder where the ingestion of gluten leads to damage in the small intestine. Inflammatory gluten-specific T cells accumulate in the small intestine and induce epithelial damage and activate cytotoxic IELs, leading to the intestinal lesions and crypt hyperplasia (Hadis et al., 2011; Meresse et al., 2012).

Paneth cells reside at the bottom of the crypts and secrete antimicrobial peptides, which together with mucus, help to keep bacteria away from the epithelial cells (Fig. 1-1). It is estimated that the jejunum is colonized by 10^4 bacteria/g intestinal content and a more dense and diverse bacterial community is present in the ileum (up to 10^8 bacteria/g content) (Zoetendal et al., 2006). Underneath the epithelium lies the lamina propria, which consists of a loosely packed connective tissue containing blood and lymph capillaries, the nervous system and most of the cells that comprise the intestinal innate and adaptive immune system. Immune cells of the lamina propria are the focus of this work as they monitor antigens within the lamina propria, such as *S. mansoni* eggs that penetrate the intestinal tissue or invading microbial antigens and are therefore described in more detail in the following sections.

Apart from the lamina propria, immune cells also reside in macroscopically visible Peyer's patches and smaller solitary isolated lymphoid tissues (SILTs), which are important structures of the gut-associated lymphoid tissue in the small intestine (Jung et al., 2010). Within these structures, aggregates of T and B cells reside within large B cell lymphoid follicles and smaller T cell areas and respond to antigen presented by dendritic cells. Peyer's patches and SILTs are specialized to recognize luminal antigen and defend against pathogens that try to invade the intestine. Luminal antigen is transported into these structures by specialized microfold cells (M cells) and presented to T cells that drive adaptive immune responses such as B cell activation and antibody production (Mabbott et al., 2013). One of the most important types of antibodies produced under homeostatic conditions in the intestine is Immunoglobulin A (IgA), which is secreted to target microbial antigens. By binding to bacteria, IgA facilitates bacterial entrapment within the lumen and the regulated uptake of luminal antigens by M cells and helps maintain intestinal homeostasis against the microbiota (Mantis et al., 2011).

Adaptive immune responses against intestinal antigens are also initiated in the mesenteric lymph nodes, which are connected to the intestine by the lymphatic system. Here, migratory immune cells, like dendritic cells, present intestinal antigen to naive T cells and initiate the appropriate immune responses. As dendritic cells are specialized to prime T cells against foreign antigens, including those derived from parasites, the mechanisms by which they prime T cells and induce immune responses will be discussed in more detail later on. Carter and

Collins already observed in 1974 that separate lymph nodes drain different segments of the intestine and identified that the upper and central node of the four to five mesenteric lymph nodes in mice drain the small intestine (Carter and Collins, 1974). They also identified that different parts of the large intestine drained into different lymph nodes located within the body cavity. The caecum and ascending colon drain into the lower of the four to five mesenteric lymph nodes, the transverse colon is drained by the pancreatic lymph nodes and the descending colon and rectum are drained by the caudal lymph node. Thus, careful distinctions between the respective lymph nodes need to be made when studying adaptive small intestinal and colonic immune responses, which reflects on work presented in this thesis that investigates and compares small intestinal and colonic immune responses against *S. mansoni* egg antigens.

The large intestine follows a similar anatomical structure as the small intestine and is encased by a muscle layer, which is responsible for intestinal peristalsis, the muscularis mucosae and submucosa, which are densely packed with nerve fibres and the serosa, which provides a thick fibrous covering and separates the intestine from the surrounding peritoneal cavity (Mowat and Agace, 2014). It extends from the small intestine to the anus and is divided into the caecum, the ascending, transverse and descending colon, and the rectum. Structurally it is a thick tube that is wider and shorter than the small intestine and reaches up to 1.5 meter in humans. In contrast to the small intestine, the colon has little or no intrinsic digestive function and its main roles are the reabsorption of water and the elimination of undigested material. As such, epithelial enterocytes have a smooth surface without microvilli, and villous structure are also missing from the colon. However, similar to the small intestine, crypts are present and stem cells develop into epithelial cells, such as enterocytes and goblet cells. Paneth cells, which secrete antimicrobial peptides in the small intestine, are not present in the colon but mucus-secreting goblet cells are much more frequent (Fig. 1-1). Two distinct layers of mucus exist in the colon, which help keep the large bacterial community of 10^{12} bacteria/g content, which mostly metabolize undigested material, at bay (Zoetendal et al., 2006). Impaired intestinal homeostasis leads to inflammatory bowel diseases where aberrant inflammatory responses are directed against these commensal bacteria and cause damage to the intestinal tissue. Crohn's disease is hereby more prevalent in the distal small intestine whereas ulcerative colitis selectively affects the colon (Knights et al., 2013).

Instead of Peyer's patches, caecal and colonic patches can be identified in the colon, which serve similar functions and are an important site for colonic T cell priming and IgA production against microbial antigens (Masahata et al., 2014). Similar to the small intestine, the colonic lamina propria harbours blood and lymph vessels, the nervous system and immune cells, which control and protect large intestinal homeostasis. The details of the innate and adaptive immune responses that exist in these tissues and maintain intestinal homeostasis or defend against foreign pathogens, such as parasites, are described in the section that follows.

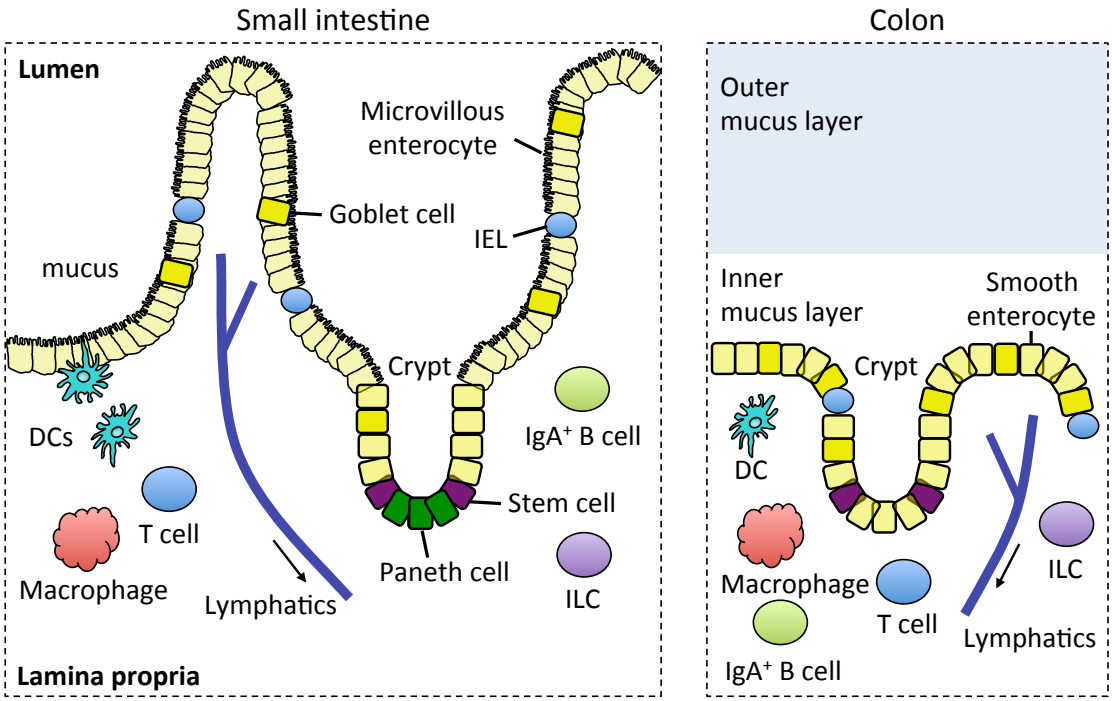

Figure 1-1. Anatomy of the small intestinal and colonic mucosa

The intestine is separated from the lumen by a single layer of epithelial cells, which are constantly renewed by stem cells in the crypts. In the small intestine microvillous enterocytes take up and digest nutrients, intraepithelial lymphocytes (IELs) provide epithelial immune defences, goblet cells produce mucus and Paneth cells release antibacterial molecules that maintains the sterility of the crypts. The colon is separated from the microbiota by two layers of mucus and its epithelium is specialized for the reabsorption of water. The lamina propria underneath the epithelium harbours cells of the innate and adaptive immune system, such as dendritic cells (DCs), macrophages, T cells, innate lymphoid cells (ILCs), and IgA^+ B cells and is connected to the draining lymph nodes by the lymphatics.

1.1.2 Innate immune cells in the intestine

Macrophages and dendritic cells play an important role in maintaining homeostatic conditions in the intestine, whereas other innate immune cells such as mast cells, basophils, eosinophils and innate lymphoid cells (ILCs) are effector cells that rapidly respond to pathogens and protect the intestine against infections. In this section we will focus on the role of these cells during homeostasis and their involvement in the defence against pathogens, especially against parasites.

Both macrophages and dendritic cells represent mononuclear phagocytes, which take up and present foreign antigens. Despite these and other similarities, like their expression of CD11c and MHCII, they develop from distinct progenitors and perform distinct functions during the immune response.
Macrophages represent the most abundant leukocyte in the healthy intestinal lamina propria. They are located in close proximity to the epithelial cell layer and are constantly replenished by $Ly6C^{hi}$ blood monocytes. $Ly6C^{hi}$ monocytes have been shown to constantly enter the colonic lamina mucosa and mature into resident macrophages, through several intermediate stages, demonstrated by adoptive transfers of monocytes into $CCR2^{-/-}$ mice, which lack blood monocytes (Bain et al., 2013). Resident macrophages are highly phagocytic and maintain intestinal homeostasis by clearing apoptotic and senescent cells locally, which is consistent with their expression of scavenging receptors such as CD36. They are also required for the proliferation of epithelial progenitors in the intestinal crypts. This was demonstrated in $Csf1^{op/op}$ mice, which lack macrophages. In these mice, epithelial wound healing after dextran sulfate sodium (DSS) treatment was severely impaired and epithelial progenitors did no longer proliferate (Pull et al., 2005). Intestinal macrophages also take up and destroy microorganisms but in contrast to macrophages elsewhere in the body do not trigger proinflammatory responses. They do not express the lipopolysaccharide (LPS) co-receptor CD14 or the IgA receptor CD89 and do not produce proinflammatory cytokines, such as Interleukin 1 (IL-1), IL-6, IL-10, IL-12 and Tumor-necrosis factor alpha (TNF-α), in response to an array of bacterial stimuli, such as LPS and flagellin, *in vitro* (Smythies et al., 2005). They do however produce large amounts of IL-10 and play an important part in intestinal tolerance by expanding and maintaining antigen-specific regulatory T cells in the intestinal mucosa. This was for example

demonstrated in CX3CR1-deficient mice, which showed impaired IL-10 production in macrophages, and did not support the proliferation of regulatory T cells in the lamina propria (Hadis et al., 2011). This correlates with the observation that spontaneous colitis develops in mice in which the IL10-IL10R regulatory axis is disrupted and intestinal homeostasis against the microbiota, predominantly against *Helicobacter hepaticus,* is no longer maintained (Kullberg et al., 2001). Thus, intestinal macrophages help maintain tissue integrity and tolerance against bacterial stimuli (Fig. 1-2). They also phagocytose invading bacteria or soluble antigen and have been shown to pass antigen on to dendritic cells that are specialized to prime naive T cells in the draining lymph nodes (Mazzini et al., 2014). Macrophages can also stimulate effector T cells in the lamina propria and thus potentiate immune responses against larger pathogens, such as parasites, which they cannot phagocytose.

In contrast to macrophages, dendritic cells are specialized to acquire antigen locally and migrate to the draining lymph nodes to prime naive T cells, that home back to the intestine to respond. Because they are the central focus of this thesis and are described in detail in the following sections, only their primary functions are presented here.

DCs develop under steady-state conditions from specific, bone marrow-derived precursors within peripheral tissues and lymphoid organs. They take up local protein antigen, process it and present distinct peptide fragments to naive T cells in the draining lymph nodes. Beyond inducing the proliferation of naive T cells that recognize the presented antigen, they also influence their differentiation into distinct T helper cells, thus shaping the immune response. Dendritic cells integrate environmental cues through a vast array of pattern recognition receptors and other danger-sensing elements and influence the strength and quality of the immune response by expressing costimulatory markers or secreting cytokines. As such, the upregulation of the costimulatory molecules CD40, CD80 and CD86 and the secretion of IL-12, drive the differentiation of proinflammatory Th1 cells (Bekiaris et al., 2014). Dendritic cells have also been shown to sample parasite antigen and are involved in the induction of Th2 responses. However, the molecular mechanisms are not well understood and it remains unclear if dendritic cells alone are sufficient to prime Th2 cells. We therefore aimed to clarify the role of intestinal DCs in the initiation of Th2 responses in this project.

Dendritic cells can furthermore imprint distinct migratory preferences onto lymphocytes and induce the expression of distinct surface molecules, such as the integrin α4β7 and the chemokine receptor CCR9, which enables these cells to home back to intestinal tissues (Iwata et al., 2004; Johansson-Lindbom and Agace, 2007). Furthermore, the idea is emerging that distinct subsets of DCs are specialized to drive specific types of immune responses, a question that is also addressed in regards to Th2 responses in this thesis.

Innate effector cells within the intestine include tuft cells, mast cells, basophils, eosinophils and innate lymphoid cells (ILCs), all of which have been shown to play a role in type 2 immune responses. Epithelial tuft cells expand rapidly after parasite infection, drive goblet cell hyperplasia, secrete IL-25, which activates IL-13 production by ILCs and promote parasite expulsion (Howitt et al., 2016; Gerbe et al., 2016). Mucosal mast cells become activated through pathogen specific antibodies such as IgE and release pre-formed mediators, such as histamine and proteases. These mediators induce mucus production and smooth muscle contraction, which helps expulse the pathogen. Mast cells can also increase vascular permeability and local blood flow and can induce the maturation of DCs and the recruitment of eosinophils and effector T cells (Urb and Sheppard, 2012). During infection eosinophils and basophils are quickly recruited from blood to the site of infection. Similar to mast cells, basophils are activated by IgE and release histamines. They can also secrete cytokines and express MHCII and some costimulatory molecules and can activate effector T cells within tissues. Eosinophils release cationic granule proteins and reactive oxygen species, which damage the pathogen. They also produce growth factors and cytokines that influence the surrounding tissue and stimulate other cells such as macrophages, mast cells and effector T cells. These innate effector cells are however not only recruited upon the infection of pathogens, such as parasites, but also during the pathologic type 2 immune responses of asthma and other allergies, and drive local inflammation (Stone et al., 2010).

ILCs, a recently identified population of innate effector cells, have rapidly been recognized as key orchestrators of immune defences at mucosal surfaces (Fig. 1-2) (Spits and Cupedo, 2012). Like other innate immune cells, ILCs lack recombined antigen receptors, are activated in the early phases of the immune response and do not generate memory immune responses. Upon activation they

secrete large amounts of cytokines and have been classified in three main subsets: ILC1s, which produce Th1 cytokines such as IFN-γ; ILC2s, which produce IL-5 and IL-13; and ILC3s, which produce IL17A and IL-22. In regards to type 2 immune responses, ILC2s have been shown to mediate resistance against parasites, such as the helminth *Nippostrongylus brasiliensis*. IL-25$^{-/-}$ mice, which lack ILC2s, displayed inefficient parasite expulsion and delayed cytokine production by Th2 cells. Upon administration of recombinant IL-25 *in vivo* ILC2s developed within 4 days and led to effective worm expulsion (Fallon et al., 2006). Similar to mast cells, basophils and eosinophils, ILC2s also play a role in type 2 mediated pathology, such as virus-mediated exacerbation of allergic asthma and drive airway hyper-reactivity even in the absence of the adaptive immune system, as demonstrated in infected Rag2$^{-/-}$ mice (Chang et al., 2011).

In conclusion, innate immune responses have evolved to rapidly respond to foreign invaders and are beneficial to defend against parasite infections. However, they are also involved in pathologic type 2 immune responses and drive the pathology of asthma and other allergies.

1.1.3 Adaptive immune cells

Adaptive immune cells (T and B cells) provide potent, antigen specific acute and memory immune responses that maintain intestinal homeostasis and protect against infections. They are present within the intestinal epithelium, the lamina propria and the mesenteric lymph nodes and are primed within the Peyer's patches or the draining lymph nodes. In this section, their role in tissue homeostasis and pathogen defence, in particular against parasites, is discussed.

Intraepithelial lymphocytes (IELs) are antigen-experienced T cells that are located within the epithelium and develop through thymic (natural) and peripheral (induced) differentiation. Natural IELs populate the gut early in life, whereas induced IELs develop from effector T cells that have responded to peripherally encountered antigens and gradually accumulate over time. Most IELs are CD8^{+}, have cytotoxic activity and can express effector cytokines, such as IFN-γ, IL-2, IL-4 and IL-17. They can kill infected epithelial cells and thus preserve the integrity of

the mucosal barrier but are not involved in the defence against multicellular organisms (Cheroutre et al., 2011).

In contrast to IELs, CD4 T cells play a more diverse role in tissue homeostasis and the defence against microbial and multicellular organisms. They are primed in secondary lymphoid organs and display an effector memory phenotype. In the steady state the most abundant CD4 T helper cell subsets in the intestine are IFN-γ and IL-2-producing Th1 cells, IL-17$^+$ Th17 cells and IL-10-producing regulatory T (T reg) cells (Zhu and Paul, 2008). Whereas proinflammatory Th1 cells that protect the host against intracellular pathogens are present at equal numbers throughout the intestine, cytotoxic CD8 T cells and antibacterial Th17 cells are more abundant in the small intestine, suggesting that microbial defence is of primary importance in this tissue. Conversely, regulatory FoxP3$^+$ Treg cells are present in higher numbers in the colon (Fig. 1-2). Tregs cells and the production of IL-10 is important in maintaining colonic homeostasis, which was demonstrated in mice where IL-10 was specifically deleted in FoxP3-expressing cells. These mice developed colonic disease and systemic sensitivity (Rubtsov et al., 2008). The mechanism by which IL-10 influences tissue homeostasis is by downregulating effector T cell responses. Colonization of germ-free mice, for example, resulted in activation and differentiation of colonic Tregs, which contributed to intestinal homeostasis. Administration of a blocking IL-10-receptor antibody during colonization however resulted in an increase in Th1 and Th17 cells and led to pathology (Geuking et al., 2011). Thus, IL-10-producing cells are crucial in regulating effector cell functions and maintaining intestinal homeostasis.

During the infection of pathogens, such as parasites, CD4 T cells are primed against parasite antigens in secondary lymphoid tissues and differentiate into Th2 cells. These Th2 cells mediate host defences against extracellular parasites but also play a role in pathologic conditions like asthma or other allergic diseases. Th2 cells produce an array of cytokines among which are IL-4, IL-5, IL-10 and IL-13. IL-4 is required for the differentiation of Th2 cells and provides a positive feedback loop (Constant and Bottomly, 1997). It also promotes IgE class switching in B cells, which can in turn activate basophils and mast cells to participate in host defence. IL-5 on the other hand is necessary to recruit eosinophils. Both mechanisms were investigated by antibody blocking experiments in nematode *Nippostrongylus brasiliensis* infected mice, where IL-4 blocking inhibited parasite-

induced IgE production and blocking of IL-5 completely suppressed blood eosinophilia and tissue infiltration (Leung et al., 2013). Th2 responses are essential for the expulsion of many parasites such as *Nippostrongylus brasiliensis* and *Trichuris muris* which is mediated directly by IL-13 production and IL-4 mediated signalling, which was demonstrated by failed parasite expulsion in IL-13 and IL-4Rα deficient mice (Urban et al., 1998). However, Th2 responses can also be harmful and promote basophil and eosinophil recruitment during allergic reactions and lead to IL-13-mediated airway hyperresponsiveness, goblet cell metaplasia and mucus hypersecretion during allergic asthma (Wills-Karp, 2004).

B cells also play an important role in intestinal homeostasis and pathogen defence and are responsible for producing antibodies that shape the humoral immune response. They acquire antigens in secondary lymphoid organs such as the Peyer's patches and the MLNs through their B cell receptors and can become activated through T cell dependent or independent activation. Most foreign proteins induce T cell dependent activation, by which follicular T helper cells recognize the antigen presented by B cells and promote B cell proliferation, immunoglobulin class switching, and somatic hypermutation, which are required to generate high-affinity antibodies. After cell activation, B cells differentiate into short-lived plasmablasts, which provide immediate protection and long-lived plasma cells and memory B cells (Pieper et al., 2013). Within the intestine, IgA is heavily produced by plasma cells residing within the lamina propria and targets microbial antigens (Fig. 2). The production of IgA is dependent on the microbiota, as germ-free mice do not produce IgA (Talham et al., 1999). By binding to microbial antigens IgA can block bacterial attachment to epithelial cells and IgA-bound bacteria become trapped within the mucus facilitating their removal (Mantis et al., 2011). Thus, IgA plays an important role in bacterial entrapment and maintains intestinal homeostasis, which was demonstrated in AID-deficient animals, which cannot undergo class switching to IgA, and display lymphoid hyperplasia of the gut and an altered microbiota (Suzuki et al., 2004a). They also produce antibodies against antigens from pathogens and the production of IgE and IgG1 antibodies are hallmarks of type 2 immune responses, both during parasite infection and pathogenic conditions, such as allergies (Mountford et al., 1994). Hereby, IgE antibodies are potent stimulators of type 2 effector cells, such as mast cells and basophils, and drive host defence or allergy pathogenesis. Thus, cells of the adaptive immune system contribute to intestinal homeostasis and

pathogen defence and provide potent, antigen specific acute and memory immune responses.

Having introduced innate and adaptive immune responses and their general involvement in parasite defence we now focus on *S. mansoni* infections and the immune responses induced by the parasite, in particular by *S. mansoni* eggs, which are the focus of this project.

	Small intestine	Colon
Anatomy	Long and coiled	Thick and short
Function	Nutrient uptake	Water reabsorption
Epithelial structure	Microvillious enterocytes	Smooth enterocytes
Commensal bacteria	10^4-10^8 bacteria/g content	10^{12} bacteria/g content
Bacterial defense	Paneth cells, mucus	Two mucus layers
GALT	Peyer's patches, SILTs	Colonic patches, SILTs
Effector T cells	IELs, Th1, Th17, CD8 T cells	IELs, Th1, fewer Th17
ILCs	ILC1s, ILC2s, ILC3s	Mostly ILC1s
Tolerogenic T cells	Few Tregs	Many Tregs
B cells	IgA^+ plasma cells	Many IgA^+ plasma cells
Mononuclear phagocytes	Macrophages, dendritic cells	Many macrophages but few DCs

Figure 1-2. Anatomical, physiological and immunological differences between the small intestine and colon in steady state

Summary of the main anatomical and physiological differences of the small intestine and colon and list of predominant immune cell populations present in both tissues in steady state.

1.2 *Schistosoma mansoni* infection and immune responses

As previously mentioned, schistosome infections have an unusually high morbidity and mortality rate compared to other helminth parasite infections, which is mostly due to damage caused by parasite eggs during chronic infection.

1.2.1 Schistosome life cycle

Infectious cercariae, which represent the free-swimming fresh water larval stage of *S. mansoni*, burrow into the skin of the human host, transform into schistosomula and travel with the blood stream to the hepatic portal vein. In the liver they mature into adult worms, which migrate into the portal vein and along the vasculature. They live as couples, with the female worm lying in the ventral groove of the male, and without treatment persist within the host for 5-7 years. Each female produces large quantities of eggs and deposits up to 300 eggs along the blood vessel endothelium on a daily basis. Around half of these eggs are able to actively cross the endothelium and basement membrane of the vein, migrate through the intervening tissue and penetrate the intestine (*S. mansoni* and *S. japonicum*) or the bladder (*S. haematobium*), causing tissue damage. The other half are carried away by the blood flow and become trapped in the liver as they cannot traverse the sinusoids. Eggs that penetrate through the intestine are excreted and miracidia hatch upon contact with fresh water and infect fresh water snails that represent the intermediate host. There they undergo asexual reproduction and develop into thousands of infectious cercariae, which are released to infect another definitive host (Fig. 1-3) (Pearce and MacDonald, 2002).

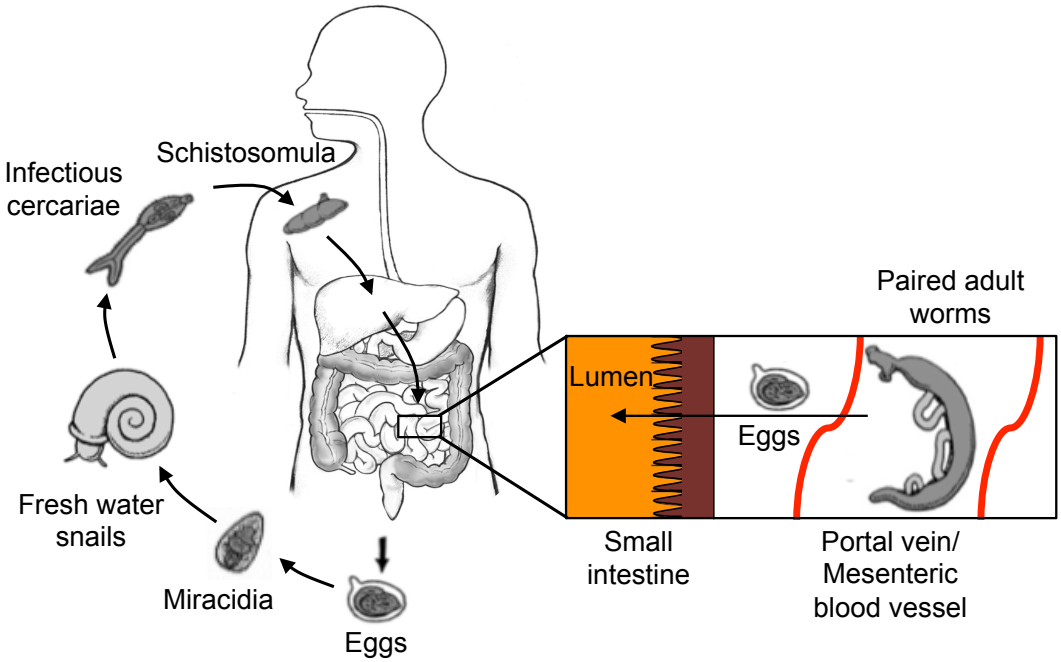

Figure 1-3. Life cycle of *Schistosoma mansoni*

Summary of the life cycle of *Schistosoma mansoni* during which infectious cercariae infect the definitive human host, and develop into adult worms. Adult females lay eggs that actively penetrate the blood vessel endothelium, the connective tissue and the intestine and are excreted. Miracidia hatch upon contact with water and infect the intermediate host, represented by several species of fresh water snails. Elements of this image were obtained from the Dr. Ating Foundation (http://www.dratingfoundation.org).

Despite its long presence within the host, natural immunity against the parasites does not occur. However, it has been observed that worm-specific IgE antibodies and partial resistance to schistosomes increase with age, while highest worm burdens and egg counts are detected in children, which could suggest that IgE antibodies confer partial immunity (Dunne et al., 1992). The treatment of schistosomiasis is so far limited to anthelmintic drugs like praziquantel, which effectively kill adult worms. Praziquantel increases calcium ion permeability of parasite cells and leads to parasite contraction and paralysis (You et al., 2013). However, praziquantel does not protect against reinfection or damage larval stages or parasite eggs. As praziquantel treatment only offers immediate relief, several experimental vaccines against worm and egg antigens have been tested. In early experiments, mice were immunized with irradiated cercariae and a single dose resulted in a 50%–70% reduction in adult worm burden. Hereby, migrating *S. mansoni* schistosomula were attacked by cell infiltrates and Th1 cells causing inflammation and entrapment and eventual killing of the larvae (Smythies et al., 1996). However, due to the strong inflammatory response induced after

immunization and the lack of suitable specific vaccine candidates, no human vaccine exists to date. It is therefore crucial to further understand the immune responses that are activated during schistosome infections and identify the underlying mechanisms, which might reveal suitable candidates for immunotherapeutic intervention.

1.2.2 Inflammatory responses characterize acute schistosomiasis

During the schistosomal life cycle the immune system mounts several immunological responses against the parasite. Skin-penetrating cercariae induce local inflammation and innate immune responses, which can trap and kill some of the infecting larvae. However, evidence is also accumulating that cercariae can modulate the immune response, by limiting dendritic cell migration and downregulating adaptive immune responses in the skin draining lymph nodes, which allows the remaining larvae to develop into schistosomula and travel with the blood stream to the liver (Jenkins et al., 2005). Clinical symptoms of early infection, known as acute schistosomiasis, peak at 6 to 8 weeks after infection and immune responses are generally mounted against adult worms. Patients suffer from an incapacitating febrile illness, known as Katayama fever, which generally occurs before eggs are detected in the stool. In these patients TNF-α is detectable in blood plasma, and peripheral-blood mononuclear cells (PBMCs) produce large quantities of inflammatory cytokines such as TNF-α, IL-1 and IL-6. Upon stimulation with worm antigen these PBMCs produce high levels of IFN-γ, but no IL-5, which is associated with Th2 responses (de Jesus et al., 2002). After the release of eggs by female adult worms, inflammatory responses are diminished and a strong Th2 response becomes detectable. It was experimentally demonstrated that IL-4$^{-/-}$ mice, which fail to develop Th2 responses, succumb to *S. mansoni* infection and experience TNF-α-mediated severe acute cachexia, hepatotoxicity and high mortality, whereas C57BL/6 mice tolerate infection and develop chronic disease (Brunet et al., 1997). Similar observations were made in mice that were infected with irradiated or single-sex cercariae, which did not produce eggs and thus did not initiate type 2 immune responses (Dunne and Cooke, 2005). It has therefore been suggested that the dampening of the initial inflammatory responses, by the onset of type 2 immune responses against schistosomal eggs, limits damage to the host and initiates chronic rather than

harmful acute disease. Type 2 immune responses however are not sufficient to destroy the parasite or the eggs, and as adult worms live within the blood vessels they cannot be expulsed by the host. Thus, chronic persistence of the worms and accumulation of trapped and encased eggs lead to a relatively high morbidity and mortality rate compared to other helminth infections.

1.2.3 *S. mansoni* egg antigens induce Th2 responses

As the induction of Th2 responses against *S. mansoni* eggs is the focus of this thesis the type 2 immune responses induced by egg antigens are presented in the following section.

With the onset of egg production hallmarks of type 2 immune responses, such as high levels of IL-4 and IL-13, IgE, eosinophilia and mastocytosis are observed in the human and murine host (Pearce and MacDonald, 2002). The induction of Th2 responses does not require adult worms and can be induced by the injection of live or non-viable eggs, water-soluble *S. mansoni* egg antigens (SEA) or excretory/secretory egg products (E/S), collected from live eggs (Cheever et al., 2002). This indicates that *S. mansoni* eggs contain and release antigens that are sufficient to prime Th2 responses. Two immunogenic proteins have so far been identified in the E/S of *S. mansoni* eggs that can mediate strong type 2 immune responses *in vitro* and *in vivo.* Omega-1 can condition bone marrow derived DCs (BMDCs) to prime Th2 cells *in vitro* and *in vivo* and has similar effects to SEA, suggesting that it is the primary antigen to induce Th2 responses (Steinfelder et al., 2009). The other identified protein, IPSE/alpha-1 triggers IL-4 and IL-13 release from murine basophils *in vitro* and *in vivo*. This release is dependent on IgE, but does not require antigen specificity, as basophils from OVA/alum sensitized mice reacted to IPSE/alpha-1, whereas basophils from $IgE^{-/-}$ animals did not (Schramm et al., 2007). In contrast to omega-1 and SEA, injection of IPSE/alpha-1 into the footpad did not induce Th2 responses in the draining popliteal lymph nodes (Everts et al., 2009), suggesting that IPSE/alpha-1 is not sufficient to induce the priming of Th2 responses but rather amplifies or maintains Th2 responses through basophils.

S. mansoni eggs have a size of 100x50 µm and the maturing miracidium is encased by a rigid insoluble eggshell. Therefore, eggs that are trapped within

tissues such as the intestine or the liver cannot be destroyed but are quickly encased by granulomas that consist of collagen fibres, CD4 T cells, eosinophils and macrophages (Fig. 1-4). These granulomas protect the surrounding tissue from secretory egg molecules and egg proteins released by dying eggs and degrade them over time. However, granulomas also damage the surrounding tissue and lead to fibrosis (Dunne and Doenhoff, 1983). Fibrosis is primarily mediated by IL-13, a key cytokine produced by Th2 cells and *S. mansoni* infected IL-13$^{-/-}$ mice do not develop severe fibrosis or hepatocyte damage (Fallon et al., 2000). Hepatic fibrosis can also be reduced by blocking IL-13 with monoclonal antibodies *in vivo* (Chiaramonte et al., 1999). Thus, different mechanisms contribute to chronic pathology and in the most serious form of chronic schistosomiasis can cause a life-threatening hepatosplenic disease, which is accompanied by severe hepatic and periportal fibrosis and portal hypertension (Dunne and Pearce, 1999).

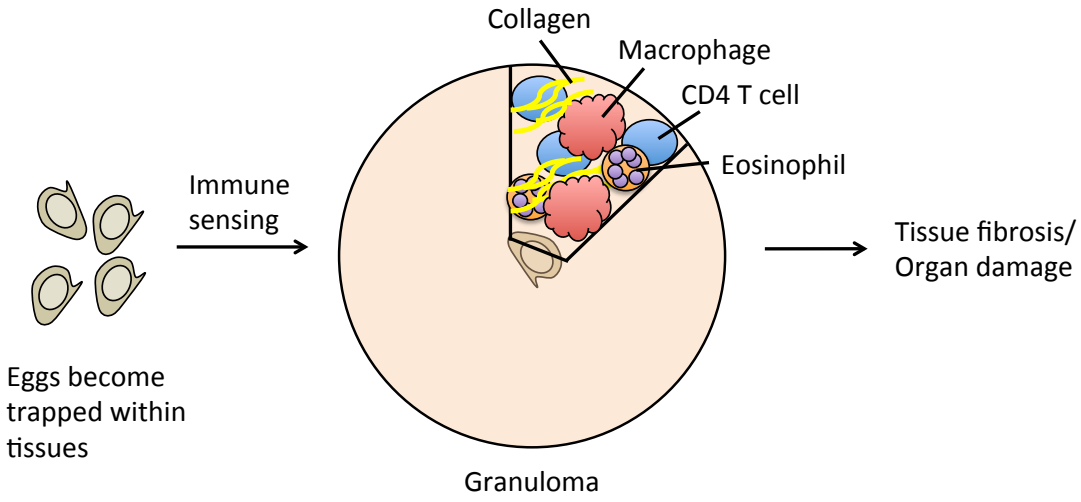

Figure 1-4. Granuloma formation leads to tissue damage

Eggs that become trapped within the intestine or the liver are quickly encased by granulomas. These consist of collagen fibres, macrophages, CD4 T cells and eosinophils and protect the surrounding tissue from molecules released by live and degrading eggs. Accumulation of granulomas however, has damaging effects on the host and can lead to tissue fibrosis and organ damage.

To prevent excessive tissue damage, Th2 responses are dampened down by regulatory mechanisms (Fig. 1-5), which is consistent with the observation that reinfection causes less reactive responses than primary infections (Dunne and Pearce, 1999). A further presence of a regulatory immune response at later stages of *S. mansoni* infection was experimentally demonstrated by Smits *et al.* They showed that experimental airway inflammation against OVA, which induces allergic Th2 responses, was enhanced 8 weeks after parasite infection, whereas airway inflammation was suppressed in mice that had been infected with schistosomes for 16 weeks (Smits et al., 2007). One of the mechanisms by which Th2 responses are controlled is by the production of IL-10. Infected IL-$10^{-/-}$ mice showed increased Th2-mediated morbidity with increased granuloma sizes and hepatic fibrosis, enhanced production of Th2 cytokines in restimulation cultures and an increased mortality during the chronic stages of infection (Hoffmann et al., 2000). IL-10 is predominantly produced by regulatory T cells, which have also been identified in granulomas of chronically infected mice (Hesse et al., 2004). Egg antigens can also directly induce the differentiation of Tregs and the injection of SEA increases the number of $Foxp3^{+}$ Tregs *in vivo* and can induce regulatory T cell development *in vitro* (Zaccone et al., 2009).

Thus, regulatory responses limit damaging effects caused by Th2 responses against trapped and encased eggs. Although the beneficial and detrimental effects of Th2 responses during *S. mansoni* infection are well characterized, less is known about how these responses are initiated and the identification of the cells responsible for inducing Th2 responses against *S. mansoni* eggs are the focus of this work.

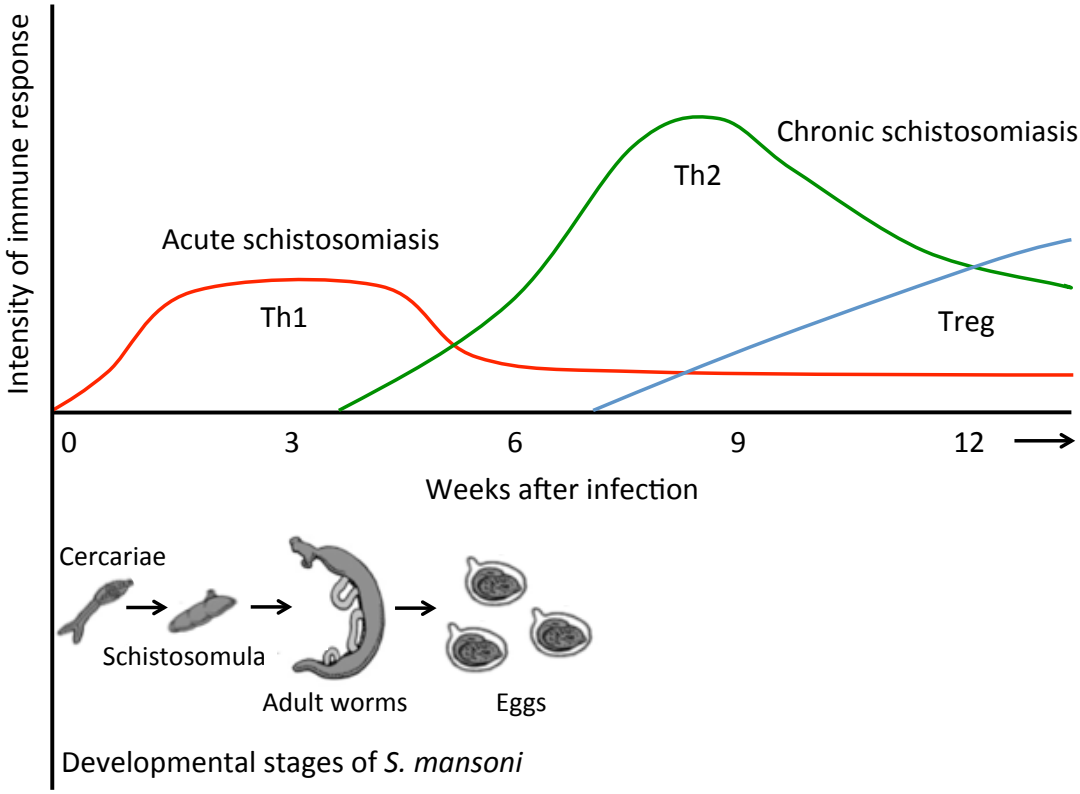

Figure 1-5. Development of the immune response during *S. mansoni* infection

During acute schistosomiasis inflammatory responses against larvae and adult worms are prominent. With the release of eggs by adult worms 6 weeks after infection, inflammatory responses are dampened and strong Th2 responses are mounted against egg antigens. With chronic persistence of infection these responses are controlled by regulatory T cells (Tregs) to limit damage to the host. Elements of this image were obtained from the Dr. Ating Foundation (http://www.dratingfoundation.org).

1.3 Dendritic cell biology with focus on the intestine

As dendritic cells bridge innate and adaptive immune responses and play an important role in responding to foreign antigens and orchestrating the appropriate immune responses, they are the focus of this work that aims to identify the cells responsible for inducing Th2 responses against *S. mansoni* eggs. To understand the biology of dendritic cells; their development, the functional characteristics of antigen uptake, DC activation, migration and antigen presentation and their role in T cell differentiation and T cell homing will be discussed.

1.3.1 Discovery and characterization of intestinal dendritic cells

Dendritic cells (DCs) were first described in 1973 by Steinman and Cohn as large stellate cells, which had a distinct phenotype from other adherent cells. Morphologically, these cells could extend and retract pseudopods, which was a unique feature of this novel cell type, and led the authors to coin the term dendritic cell. Steinman and Cohn identified these cells in preparations from the spleen, mesenteric lymph nodes, cervical nodes and Peyer's patches, but not the thymus and bone marrow and deduced that they preferentially reside in peripheral lymphoid organs. Though present in only small numbers, ranging from 0.1-1.5% of all nucleated cells, they could identify these cells in several rodent species and other mammals (Steinman and Cohn, 1973).

They also recognized that DCs were morphologically different from macrophages but were not able to identify these cells in the intestine. Indeed, DCs can be easily identified in peripheral lymph nodes, using more recent technologies like flow cytometry, by their expression of the cell surface markers CD11c and MHCII. However, as they share these markers with other cells like macrophages, their identification in peripheral tissues, like the intestine, is more complex (Cerovic et al., 2014). Until recently it was believed that the expression of the fractalkine receptor CX3CR1 defined mucosal tissue resident DCs and the expression of the integrin CD103 defined migratory DCs. Further experiments however demonstrated that $CX3CR1^{hi}$ cells represent sessile macrophages, which are influenced by the growth factor CSF1 and in the intestine differentiate from blood monocytes, whereas $CD103^{+}$ cells represent DCs, which respond to the growth factor Flt3L and migrate to the MLNs (Varol et al., 2009; Bogunovic et al., 2009).

Since then it has become clear that the expression of CX3CR1 on intestinal macrophages and CD103 on intestinal DCs are not sufficient to fully separate these populations. CX3CR1 is also expressed by a subset of intestinal DCs, although at lower levels compared to intestinal macrophages, and CD11b-expressing DCs have recently been identified in intestinal lymph and the intestine, which need to be carefully distinguished from CD11b-expressing macrophages (Cerovic et al., 2013; Scott et al., 2016). For an accurate distinction between macrophages and DCs in the murine intestine the expression of CD64, F4/80 or MerTK can be used to exclusively define macrophages (Gautier et al., 2012). This strategy has been adopted in this project and intestinal macrophages were characterized by their expression of MHCII, CD11c and CD64, whereas dendritic cells were defined as MHCII and CD11c-expressing $CD64^-$ cells.

It is important to accurately differentiate these two cell types as they serve distinct functions. Macrophages are sessile highly phagocytic cells that do not migrate to the mesenteric lymph nodes and cannot prime naive T cells, but are essential for tissue remodelling and host defence (Bain and Mowat, 2014). DCs on the other hand shape the immune response by taking up antigen in the periphery, migrating in lymph and priming naive T cells in the draining lymph nodes, which differentiate into effector T cells that respond to the antigen (Schulz et al., 2009). Many important discoveries that have advanced our understanding of dendritic cell biology were made in the Steinman laboratory, which after the discovery of DCs, identified that they were present in the periphery in an immature state, matured upon antigen uptake and presented antigen via MHCII, which drove potent T cell proliferation (Schuler and Steinman, 1985; Romani et al., 1989). The identification of the growth factors CSF2 (GM-CSF) (Inaba et al., 1992a; b) and Flt3L (Karsunky et al., 2003; Waskow et al., 2008), necessary for the development of dendritic cells, which is described in the following section, enabled the generation of dendritic cells *in vitro*, which facilitated the further characterization of these cells. Insights, like the importance of costimulatory markers and secreted cytokines in shaping T cell differentiation, which are described in a later section, were thus identified.

1.3.2 Development of dendritic cells and their subsets

DCs develop from hematopoietic stem cell progenitors and are constantly replenished in peripheral tissues and peripheral lymphoid organs. Hematopoietic stem cells differentiate into committed macrophage and dendritic cell progenitors (MDPs) in the bone marrow, which can give rise to monocytes, macrophages and resident spleen DCs *in vivo* and can differentiate into macrophages and DCs *in vitro* (Fogg et al., 2006). MDPs transition into common dendritic cell progenitors (CDPs) in the bone marrow, which have the potential to give rise to splenic plasmacytoid DCs (pDCs) and $CD8\alpha^+$ and $CD8\alpha^-$ dendritic cells, *in vivo* and *in vitro*, but can no longer develop into monocytes and macrophages (Naik et al., 2007). Committed precursors of DCs (pre-DCs), which can no longer differentiate into plasmacytoid DCs, then enter the circulation. They travel to high endothelial venules through which they enter the lymph nodes (LN) and have been shown to give rise to “LN resident” DC populations, which develop from circulating progenitors rather than migrate from the periphery, and splenic DCs (Liu et al., 2009). These DCs can be divided into two distinct subsets; $CD8\alpha^+$ and $CD11b^+CD8\alpha^-$ DCs, which both express MHCII and CD11c (Fig. 1-6) (Vremec and Shortman, 1997). $CD8\alpha^+$ DCs highly express BATF3 and are no longer detectable in the spleen of $BATF3^{-/-}$ mice (Hildner et al., 2008). $CD8\alpha^+$ DCs also depend on IRF8 and $CD8\alpha^+$ DCs are absent from $IRF8^{-/-}$ mice. In contrast, IRF4, but not IRF8, is highly expressed in $CD11b^+CD4^+CD8\alpha^-$ DCs and these cells are found at reduced frequencies in the spleens of $IRF4^{-/-}$ mice and can not be generated *in vitro* from $IRF4^{-/-}$ bone marrow cells (Suzuki et al., 2004b).

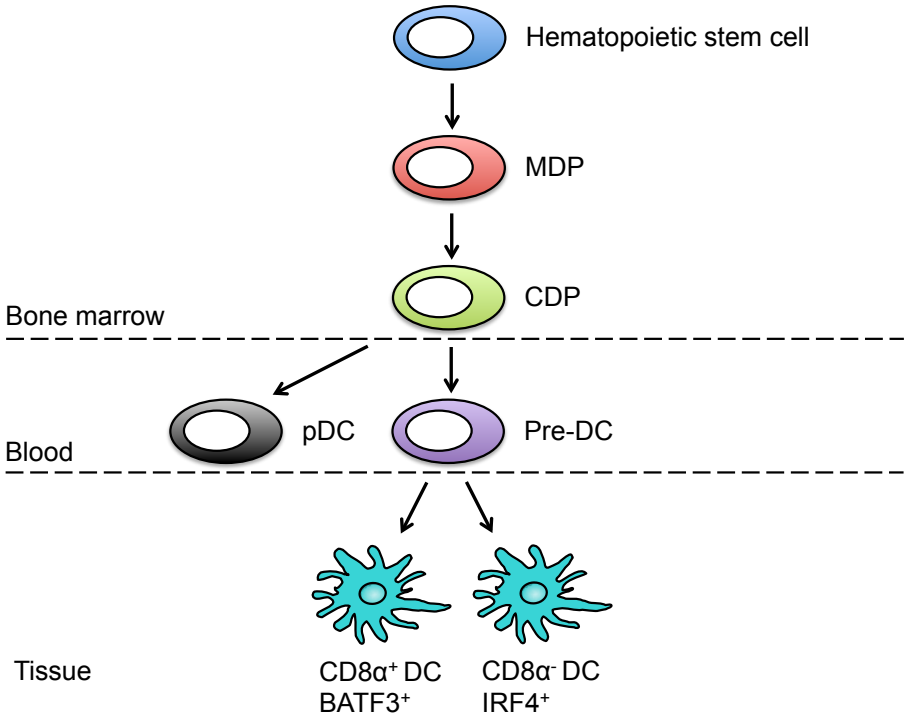

Figure 1-6. Differentiation of dendritic cells

DCs develop from hematopoietic stem cells in the bone marrow, which differentiate into macrophage and dendritic cell progenitors (MDPs) and common dendritic cell progenitors (CDPs). CDPs give rise to plasmacytoid DCs and committed precursors of DCs (pre-DCs), which enter the circulation. In the tissue pre-DCs then differentiate into the two main lineages of $CD8\alpha^+$ and $CD8\alpha^-$ dendritic cells.

Apart from giving rise to DCs in secondary lymphoid tissues, like lymph nodes and the spleen, DC progenitors also give rise to DCs in peripheral tissues. Within the intestinal lamina propria (LP) adoptive transfers of MDPs into diphtheria toxin-treated CD11c-diphtheria toxin receptor (DTR) mice, which lack CD11c-positive cells, showed that they can give rise to $CX3CR1^{hi}$ macrophages and CD103-expressing dendritic cells. Furthermore, transferred Ly6C monocytes developed into intestinal macrophages and upregulated CX3CR1, whereas the transfer of pre-DCs gave rise to DCs within the LP (Varol et al., 2009). Therefore, intestinal LN and tissue resident DCs derive from the same bone marrow precursors. Within the intestinal mucosa DCs are located diffusely in the lamina propria and gut-associated lymphoid tissues like Peyer's patches and smaller lymphoid aggregates. Within the lamina propria DCs can be divided into four subsets based on their expression of the integrins CD11b and CD103. All four subsets represent bona fide DCs that migrate from the lamina propria to the draining lymph nodes, prime T cell responses and respond to the dendritic cell specific growth factor Flt3L (Cerovic et al., 2013). $CD11b^-CD103^+$ DCs (described herein as $CD103^+$

DCs), which also express CD8α, are related to LN resident $CD8\alpha^+$ DCs as they express BATF3, which is critical for their development and also depend on IRF8 (Edelson et al., 2010). $CD11b^+CD103^+$ "double positive" (DP) DCs share characteristic elements with LN resident $CD11b^+CD8\alpha^-$ DCs. They share the expression of CD11b with LN resident $CD11b^+CD8\alpha^-$ DCs but have also acquired CD103 expression. Similar to LN resident $CD11b^+CD8\alpha^-$ DCs, they express IRF4 and fewer DP DCs are observed in the LP and MLNs of $IRF4^{f/f}$ CD11c-cre-positive mice, which have IRF4 deficient CD11c-expressing cells (Persson et al., 2013b). It has been suggested that IRF4 impacts mucosal DCs by regulating their survival, as intestinal IRF4-deficient DP DCs were more prone to enter apoptosis *in vitro* and displayed enhanced Annexin V-staining *in vivo*, which identifies cells undergoing apoptosis (Persson et al., 2013b). Similarly, IRF4-deficient $CD11b^+$ DCs from the lung show enhanced mitochondrial fragmentation, which is another indicator for cell apoptosis (Schlitzer et al., 2013). It has also been suggested that the signalling receptor Notch2 controls the terminal stage of intestinal DP DC differentiation. Both splenic $CD11b^+$ and intestinal DP DCs do not develop in $Notch2^{f/f}$ CD11c-cre mice as well as other transgenic lines that are deficient in Notch2 signalling (Satpathy et al., 2013). The development of intestinal $CD11b^+CD103^-$ (described herein as $CD11b^+$ DCs) DCs and $CD11b^-CD103^-$ "double negative" (DN) DCs are unfortunately less well characterized as most studies have not differentiated them from intestinal macrophages or further distinguished intestinal total $CD103^-$ DCs by the expression of CD11b.

1.3.3 Antigen uptake by intestinal dendritic cells

One of the characteristic features of DCs is the uptake of antigen in the periphery, which they fragment and present to naive T cells in the draining lymph nodes.
Cellular uptake of antigen by DCs has been the focus of many studies, which range from simple *in vitro* experiments to complex multiphoton studies *in situ*. Within the intestine most foreign antigens are present within the luminal content, which is physically separated from the lamina propria by a layer of mucus and the epithelium. One of the mechanisms by which antigen is transported across the epithelium is by microfold cells (M cells), which are located within the epithelial layer of Peyer's patches. They are highly specialized for the phagocytosis and transcytosis of luminal antigen and are in close contact with lymphocytes,

macrophages and dendritic cells to enable the efficient transfer of lumenal antigens (Mabbott et al., 2013). In the absence of M cells (Kanaya et al., 2012) or in models where antigen sampling by M cells is impaired (Hase et al., 2009), antigen-specific T-cell responses in the Peyer's patches of mice, which were orally infected with pathogenic bacteria were significantly reduced. Furthermore, as demonstrated by two-photon microscopy of Peyer's patch explants, dendritic cells can extend their dendrites through M cell-specific transcellular pores and directly sample luminal antigen and pathogenic bacteria from the lumen (Lelouard et al., 2012).

Several studies have claimed that lamina propria DCs can directly take up luminal antigen and extend their dendrites into the intestinal lumen by forming tight-junction-like structures with intestinal epithelial cells (Rescigno et al., 2001; Niess et al., 2005). However, early studies identified these tissue DCs by their expression of CX3CR1, which we now know defines macrophages, rather than DCs. More recent work has indeed revealed that CX3CR1^{hi} macrophages are more efficient in taking up luminal antigen compared to LP DCs. This was demonstrated by injecting fluorescently labelled OVA protein or fluorescent bacteria and visualized using two-photon microscopy (Farache et al., 2013). DCs have been shown to probe the basal surface of the epithelium in steady state but do not extend dendrites into the lumen. Furthermore, low molecular weight soluble antigens are preferentially transported across the epithelium by goblet cells and delivered to LP DCs via goblet-cell-associated antigen passages (McDole et al., 2012). It has also been suggested that macrophage-acquired antigen is accessible to LP DCs via gap junctions (Mazzini et al., 2014).

Thus, under steady state conditions luminal antigens are acquired by DCs through accessory cells, like M cells, goblet cells or macrophages, which transport the antigen from the intestinal lumen to the Peyer's patch or the lamina propria. Antigen is then sampled by DCs and presented to naive T cells either within the Peyer's patch or in the draining lymph nodes. In this project *S. mansoni* eggs are delivered into the intestinal lamina propria to represent penetrating and trapped eggs within the tissue. We therefore focused on the antigen-dependent activation of LP DCs and their migration to the MLNs in the following section.

1.3.4 Dendritic cell activation and migration

Early studies in the Steinman laboratory revealed that Langerhans cells (LCs), a type of skin dendritic cell, reside as immature cells in the skin, where they capture local antigen and migrate to the draining lymph nodes (Macatonia et al., 1987). LCs could also be matured *in vitro* and possessed the capacity to process and present antigen, by displaying it on the cell surface via MHCII and upregulated costimulatory molecules during their maturation. However, they could no longer present novel antigen once they were fully matured (Romani et al., 1989). Several pathogen-associated products, like LPS, and other danger signals can induce DC maturation and migration. The uptake of these antigens activates DCs by binding to pattern recognition receptors, such as Toll-like receptors, which can recognize a wide range of extracellular and intracellular motifs from bacterial, viral, fungal and parasitic pathogens (Joffre et al., 2009).

In the intestine TLR activation leads to DC activation and the upregulation of costimulatory molecules and cytokines. It was shown that murine $CD103^+$ LP DCs express TLR3, TLR7 and TLR9 and produce IL-6 and IL-12p40, following TLR ligand stimulation (Fujimoto et al., 2011). DP DCs on the other hand expresses TLR5 and TLR9 and produce proinflammatory cytokines such as IL-6 and IL-12 in response to flagellin and CpG (Uematsu et al., 2008). TLR activation also contributes to the migration of DCs from the lamina propria to the draining lymph nodes. This was first shown by MacPherson *et al.* in lymph from lymphadenectomized rats. Intravenous injection of endotoxin greatly increased the migration of DCs within 6 hours and reached a maximum output of 10 fold compared to steady-state migrating DCs. The increase in DC migration was hereby completely dependent on TNF-α and treatment with blocking antibodies prevented enhanced DC migration (MacPherson et al., 1995). The oral administration of R848, a TLR7/8 agonist, (Yrlid et al., 2006) or soluble flagellin (Flores-Langarica et al., 2012), which activates TLR5, also induces the migration of intestinal DCs from the lamina propria to the MLNs.

Even without the external administration of TLR ligands intestinal DCs constitutively migrate through the lymphatic system to the MLNs and were first identified by Pugh *et al*. in lymph from mesenteric lymphadenectomized rats (Pugh

et al., 1983). We now know that all four subsets of murine intestinal DCs migrate in lymph of mesenteric lymphadenectomized mice under steady state conditions. Their relative frequencies resemble those found in the lamina propria and they can be distinguished from LN resident DCs in the MLN by their higher expression of MHCII (Cerovic et al., 2013). Experiments using two-photon microscopy of MLN-afferent lymphatics revealed that CD11c-EYFP cells readily migrate under steady state conditions and in increased numbers after R848 administration, whereas CX3CR1$^{+/GFP}$ cells could not be identified, which clearly indicates that DCs migrate from the LP to the MLNs, whereas macrophages do not (Schulz et al., 2009).

The migration of LP DCs is essential for the induction of appropriate immune responses in the MLNs. In the case of oral tolerance, tolerance does not occur in mesenteric lymphadenectomized animals or transplanted MLNs that are no longer connected to the intestinal lymphatics, indicating that cell migration from the LP is critical. Indeed, expression of the chemokine receptor CCR7 on DCs is necessary for the induction of immune responses, such as oral tolerance, which was not induced in CCR7-deficient mice (Worbs et al., 2006). It has been shown in the skin and the intestine that the deletion of CCR7 does not affect the number of DCs in the periphery but impairs their migration, as they can no longer enter lymphatic vessels. Skin migrating DCs directionally move along the CCL21 gradient and bind to CCL21, via CCR7, before entering the lymphatic system (Tal et al., 2011). Correspondingly, deletion CCL21 also leads to defects in LP DC migration and an impaired induction of oral tolerance (Worbs et al., 2006). However, other immune responses, such as contact hypersensitivity, autoimmune encephalitis and allergic asthma, develop in the absence of CCR7 or its ligands. In these models immune responses were delayed but did occur, suggesting that cells which did not depend on CCR7 for their migration, or passive transport of antigen in lymph or blood were sufficient to drive these immune responses (Förster et al., 2008). Thus, the migration of activated conventional DCs to the mesenteric lymph nodes is especially important during steady state conditions and is essential to maintain tissue homeostasis in the intestine by promoting oral tolerance against benign antigens.

1.3.5 Antigen presentation and induction of CD4 T cell differentiation by intestinal dendritic cells

On reaching the lymph nodes, DCs can present antigens to naive T cells via MHC class I or MHC class II. MHCI molecules bind peptides generated from the degradation of cytosolic proteins and predominantly display intracellular proteins to cytotoxic CD8 T cells. Exogenous proteins that after cellular uptake are released into the DC cytoplasm, can also be presented via MHCI, a phenomenon known as cross-presentation. However, most exogenous antigens are presented via MHCII. Antigens are hereby degraded within the DC and peptides are loaded onto MHC molecules through the endocytic pathway. MHC/antigen complexes are then transported to the plasma membrane and presented on the cell surface. During DC maturation more MHCII/antigen complexes are present on the plasma membrane and are necessary to prime CD4 T cells (Villadangos et al., 2005). The MHCII/antigen complex is recognized by a particular T cell clone by interacting with the corresponding T cell receptor and CD4, which leads to T cell activation. The second signal that DCs provide is the expression of co-stimulatory or co-inhibitory molecules, which determine the functional outcome of T cell receptor signalling and either stimulate or inhibit CD4 T cells (Chen and Flies, 2013). Activated T cells then produce IL-2 and proliferate. However, a third signal is required for the differentiation of T cells into distinct helper subsets, which DCs can provide by producing essential cytokines.

Four major groups of CD4 T helper cell subsets (Th1, Th2, Th17 and Treg cells) with distinct properties and functions have been identified (Fig. 1-7). In 1993 Ken Murphy and colleagues were able to differentiate naive CD4 T cells into IFN-γ producing Th1 cell *in vitro*. This differentiation required antigen presentation and stimulation with heat-killed *Listeria,* which induced IL-12 production, critical for Th1 differentiation (Hsieh et al., 1993). IL-12 induces minimal levels of IFN-γ production in T cells. T cells then upregulate the key transcription factor T-bet and develop into fully differentiated Th1 cells, which mainly produce IFN-γ (Szabo et al., 2000). IFN-γ then acts in a positive feedback loop by driving the differentiation of further Th1 cells. It is also a major effector cytokine of inflammatory responses and mediates immune responses against intracellular pathogens like viruses and bacteria, by for example activating microbicidal activity in macrophages (Kaiko et al., 2008).

The second subset of $CD4^+$ T helper cells are Th2 cells, which mediate host defences against extracellular parasites but also play a role in pathologic conditions like asthma or other allergic diseases. These cells were first identified in 1990 when naive CD4 T cells were stimulated with the T-cell receptor ligand anti-CD3, IL-2 and IL-4 *in vitro* (Le Gros et al., 1990). Their most pronounced feature is the production of IL-4 and their *in vitro* differentiation requires signalling through the IL-4 receptor, Stat-6 and GATA-3. Th2 cells produce an array of cytokines among which are IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25 (Licona-Limón et al., 2013). IL-4 is a positive feedback cytokine and is required for the differentiation of further Th2 cells. It can be provided by already differentiated Th2 cells, by other effector cells such as mast cells, basophils or ILC2s, but is also produced in low amounts by naive CD4 cells upon TCR activation *in vitro* (Yamane et al., 2005). IL-4 is responsible for IgE class switching in B cells, which can in turn activate basophils and mast cells. IL-5 is involved in the recruitment of eosinophils, which together with basophils and mast cells participate in host defences against parasites or pathologic allergic conditions. Furthermore, Th2 derived IL-10 can inhibit Th1 cell proliferation *in vitro* and suppress proinflammatory responses by dendritic cells, including the secretion of IL-12 *in vitro*, and their capacity to prime Th1 cells *in vivo* (De Smedt et al., 1997). Th2 responses are also essential for the expulsion of many parasites such as *N. brasiliensis* and *T. muris*, which is mediated directly by IL-13 production and IL-4 mediated signalling (Urban et al., 1998). Thus, Th2 cells play a key role in the defence against multicellular organisms, such as parasites, but are also involved in pathologic allergic conditions.

A more recently identified T helper cell population are Th17 cells. They were first identified by their unique secretion of IL-17 and can be differentiated from naive T cells through TCR activation and the addition of IL-6 and TGF-β *in vitro* (Mangan et al., 2006). They express RORγt and produce IL-21 and IL-23, which are important in Th17 cell amplification and stabilization. Th17 responses have been found to act against many extracellular bacteria and fungi and drive many organ-specific autoimmune diseases (Weaver et al., 2006). The development of Th17 cells has been linked to Treg differentiation, as both cell populations require TGF-β for their development. Natural regulatory T cells (Tregs) develop in the thymus and play a critical role in self-tolerance. Induced Tregs are primed in the periphery and are involved in peripheral tolerance and immune regulation, such as oral

tolerance against benign antigens. Foxp3 has been reported as a master transcriptional regulator of these cells, which produce TGF-β and IL-10 and the transfer of Foxp3^{+} cells has been shown to ameliorate many immunological and autoimmune conditions, such as allograft rejection and the onset of colitis (Joffre et al., 2008; Read et al., 2000).

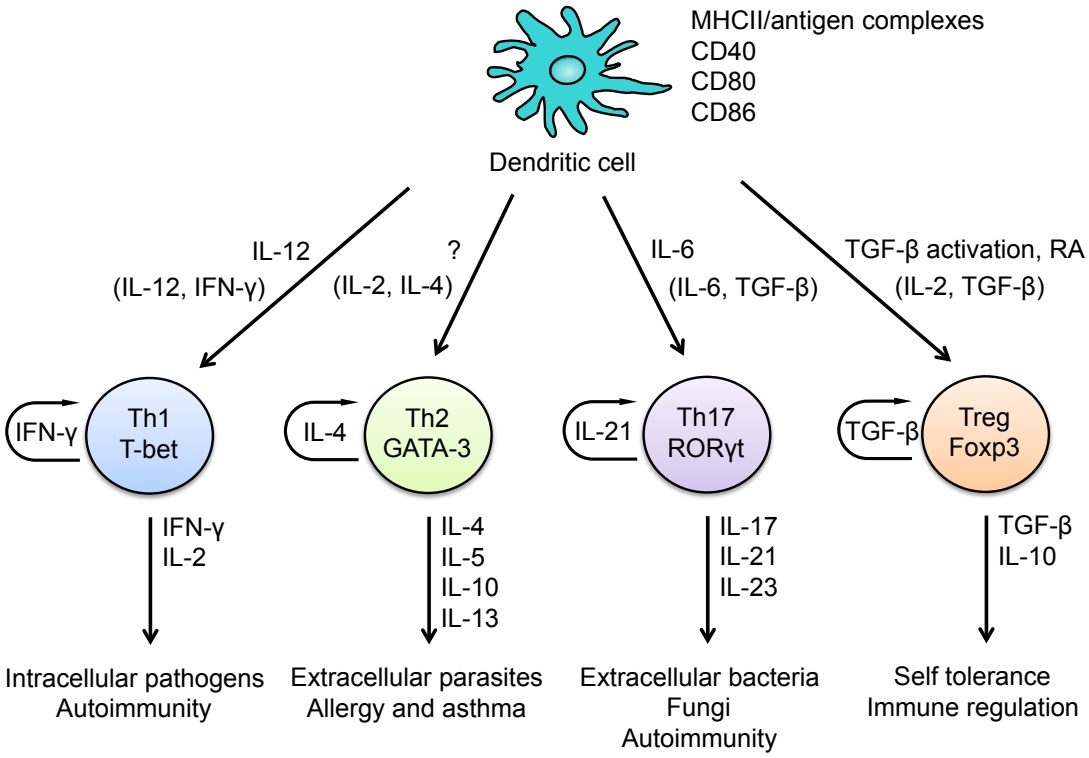

Figure 1-7. T helper cell differentiation

Activated dendritic cells play a crucial role in differentiating naive CD4 T cells into particular T helper cell populations. The cytokines that are produced by dendritic cells to drive this differentiation are summarized and the cytokines used to differentiate these T helper cell subsets from naive T cells *in vitro* are shown in brackets. The four main T helper cell populations of Th1, Th2, Th17 and Treg cells are illustrated and their characteristic transcription factors, positive feedback cytokines, effector cytokines and typical role in immunity are described.

The differentiation of these T helper cell subsets requires antigen presentation and cytokine production by dendritic cells. Many studies have identified the necessary signals *in vitro* but data are also emerging that specific DC subsets are involved in the differentiation of certain T helper cell populations *in vivo*.

In regards to the four intestinal DC subsets, CD11b^{+}CD103^{+} DP DCs from the small intestinal lamina propria (SI LP) could induce Th1, Th17 and Foxp3^{+} regulatory T cells in *in vitro* cocultures (Fujimoto et al., 2011). Similar responses have also been observed *in vivo.* IRF4$^{f/f}$ CD11c-cre-positive mice, which have

reduced numbers of SI LP DP DCs, display reduced numbers of Th17 cells in the intestine, whereas numbers of IFN-γ^{+} and Foxp3^{+} were comparable to control animals. Furthermore, transferred OT-II T cells could not differentiate into Th17 cells after immunization in these mice *in vivo.* Hereby, IRF4-deficient MLN DCs were specifically impaired in the production of IL-6 and IL-6$^{-/-}$ mice failed to support Th17 cell differentiation *in vivo*. It was therefore suggested that IL-6 is produced by DP DCs, which is required to drive Th17 differentiation in the MLNs (Persson et al., 2013b). Total CD103-expressing DCs from the SI LP and MLNs are also particularly potent in converting naive T cells into Foxp3^{+} Tregs *in vitro*. This conversion is mediated by TGF-β and retinoic acid acts as an important cofactor during Foxp3 induction (Coombes et al., 2007). Unfortunately, total CD103-expressing DCs were not separated into DP and CD103^{+} DCs in this study. However, only DP DCs express Raldh2, which converts Vitamin A into retinoic acid, and induce Foxp3^{+} regulatory T cells *in vitro* (Fujimoto et al., 2011). These cells can also activate TGF-β, through their expression of the integrin αvβ8, and it is therefore likely that DP DCs are specialized in the induction of Tregs, and therefore play an important role in maintaining oral tolerance against harmless antigens from the intestine (Pabst and Mowat, 2012).

CD103^{+} single positive SI LP DCs on the other hand have been shown to drive IFN-γ responses *in vitro*, although to a lesser extent than DP DCs. Compared to DP DCs, CD103^{+} single positive DCs did not express Raldh2 and could not induce Foxp3^{+} regulatory T cell *in vitro* (Fujimoto et al., 2011). Furthermore, CD103^{+} DCs from intestinal lymph have been shown to poorly prime CD4 T cells *in vitro* but preferentially prime CD8 T cells (Cerovic et al., 2013). Correspondingly, they have been identified as being uniquely able to cross-present endogenous antigen to cytotoxic CD8 T cells both *in vitro* and *in vivo* (Cerovic et al., 2015).

Little is known about the function of intestinal CD11b^{+} and DN DCs in driving immune responses. Combined as CD103^{-} DCs, they have been shown to express high levels of IL-12 and IL-23 following overnight culture with anti-CD40 antibodies and produce TNF-α and IL-6 after LPS stimulation *in vitro* (Coombes et al., 2007). When isolated from lymph they could prime CD4 and CD8 T cells *in vitro* (Cerovic et al., 2013), suggesting that they may be involved in inflammatory immune responses and the priming of Th1 and Th17 cells.

To date the colon, which harbours lower numbers of DCs, has been largely overlooked in functional studies of DC biology. On a phenotypic level we know that colonic $CD11b^+$ DCs outnumber $CD11b^+CD103^+$ DP DCs (Denning et al., 2011), and that colonic DCs migrate to distinct colonic lymph nodes (Houston et al., 2016), but little is known about their role in driving immune responses in the colon. Recently, a role for $CD11b^+$ DCs was suggested in colonic tolerance. Similar to oral tolerance, colonic tolerance was induced by the intracolonic administration of OVA protein, which induced proliferation and Treg differentiation of transferred OT-II cells in the colonic draining lymph nodes *in vivo*. Colonic tolerance was not affected by the deletion of colonic $CD103^+$ DCs using $BATF3^{-/-}$ mice, suggesting that colonic $CD11b^+$ DCs are sufficient to drive colonic tolerance (Veenbergen et al., 2016).

Taken together, these data indicate that intestinal dendritic cells have subset specific and tissue specific roles in T cell priming and differentiation (Fig. 1-8). However, the signals that are produced by dendritic cells to influence T cell differentiation *in vivo* are only just emerging and further work is required to elucidate their role in Th2 differentiation.

1.3.6 Intestinal dendritic cells promote gut homing of T and B cells

Apart from driving T cell responses, intestinal DCs also influence the homing of MLN primed T cells to the intestine. Hereby, effector/memory CD4 T cells primed in the MLNs rapidly express the chemokine receptor CCR9 and α4β7. This expression is only observed on MLN primed T cells, as cells primed in inguinal lymph nodes express the adhesion molecule P-selectin ligand but only low levels of α4β7 (Campbell and Butcher, 2002). CCR9 binds to CCL25, which is produced by epithelial cells within the small intestine and α4β7 binds to MADCAM1 on vascular endothelial cells, enabling CCR9 and α4β7 expressing cells to preferentially home to the small intestine (Kunkel et al., 2000).

The importance of intestinal dendritic cells in providing the signals necessary to drive CCR9 and α4β7 expression on T cells was exemplified by the observation that gut homing was impaired in CCR7-deficient mice. Transferred OT-I T cells did not upregulate CCR9 and α4β7 in the MLNs of $CCR7^{-/-}$ mice and did not migrate to

the SI LP after oral OVA administration. Furthermore, total CD103-expressing DCs from the MLN could more efficiently induce CCR9 and α4β7 expression of OT-I and OT-II T cells *in vitro*, compared to total $CD103^-$ DCs (Johansson-Lindbom et al., 2005). One of the signals provided by DCs that drive α4β7 expression is the Vitamin A metabolite retinoic acid. Small intestinal DCs, in particular DP DCs, can efficiently generate retinoic acid through the enzyme Raldh2 (Fujimoto et al., 2011). Cocultures of MLN DCs and the administration of retinol enhanced the α4β7 expression of anti-CD3/anti-CD28 stimulated T cells *in vitro*. This expression was suppressed by the retinal dehydrogenase inhibitor citral and the retinoic acid receptor antagonist LE135. Upon transfer, these T cells preferentially homed to the small intestine, Peyer's patches and MLNs *in vivo.* In the absence of retinoic acid, accomplished by feeding mice with a Vitamin A deficient diet, the expression of α4β7 and small intestinal homing of memory/activated T cells was also reduced (Iwata et al., 2004). CCL25 and CCR9 interaction also directs the migration of IgA^+ plasmablasts to the small intestine. The involvement of dendritic cells in the upregulation of CCR9 on plasmablasts however has yet to be demonstrated (Kunkel et al., 2000).

In regard to lymphocyte homing to the colon, the interaction of CCL28 and CCR10 have been shown to play an important role. CCL28 is expressed on epithelial cells in the colon and rectum, and CCR10 is expressed by circulating and mucosal IgA^+ plasmablasts (Kunkel et al., 2003). Another important molecule that is involved in T cell homing into the colon is GPR15. It has been demonstrated that GPR15-deficient mice were prone to develop more severe large intestinal inflammation, which was rescued by the transfer of GPR15-sufficient Tregs (Kim et al., 2013). The involvement of colonic dendritic cells in driving the expression of CCR10 or GPR15 has yet to be demonstrated and it remains to be determined what cells and factors influence their expression.

G protein coupled receptors, such as CCR9, CCR10 and GPR15, are therefore important for lymphocytes to home to different regions of the intestine. The expression of CCR9 and α4β7 drive T and B cell homing to the small intestine and intestinal DCs in the MLN influence the expression of α4β7 by generating retinoic acid. Thus, intestinal lamina propria DCs provide a critical link between the innate and adaptive immune system, by sampling intestinal antigen, migrating through the lymphatic system, inducing the appropriate T cell responses within the MLNs

and instructing these cells to home to the intestine to serve their immunological functions. Furthermore, the idea is emerging that specific DC subsets are specialized to perform different roles in T helper cell differentiation and tissue homing and one aim of this thesis is to determine if specific intestinal DC subsets are specialized to prime Th2 cells and drive Th2 responses against *S. mansoni* egg antigen. Experiments that have demonstrated that dendritic cells are able to actively promote Th2 differentiation are therefore discussed in the following section.

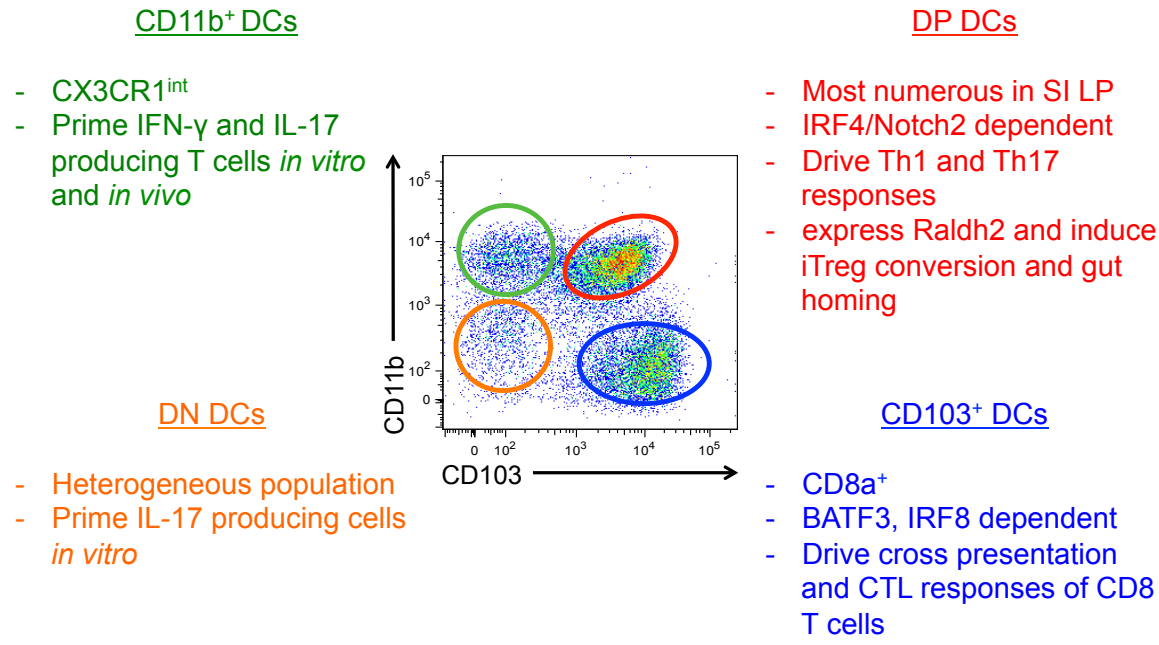

Figure 1-8. Functions of intestinal DC subsets

Summary of the most characteristic phenotypes and functions identified for intestinal DCs, which can be separated into four subsets by their expression of the integrins CD11b and CD103.

1.4 Involvement of dendritic cells in driving Th2 responses

1.4.1 Dendritic cells actively drive Th2 immunity

Multiple studies have shown that DCs are essential for priming Th2 responses. The conditional ablation of DCs *in vivo* using CD11c-DTR mice has facilitated the study of DC-dependent immune responses (Saito et al., 2001). Upon administration of diphtheria toxin $CD11c^+$ cells are eliminated, which impact T cell priming and differentiation, including the induction of type 2 immune responses. In the case of allergen-induced asthma, endogenous or adoptively transferred Th2 cells did not produce IL-4, IL-5 or IL-13 after the ablation of $CD11c^+$ cells. Eosinophilic inflammation and Th2 cytokine secretion were restored by adoptive transfer of DCs into these animals. However, the transfer of alveolar macrophages, which are also depleted after diphtheria toxin administration, did not restore the Th2 phenotype, clearly indicating that DCs were necessary to drive allergic immune responses (van Rijt et al., 2005). Similarly, alum induced Th2 responses did not develop in CD11c-DTR mice. In these experiments, alum acted as a Th2 adjuvant for OVA antigen, which was transported by monocyte–derived and conventional DCs to the draining lymph nodes and presented to adoptively transferred OT-II CD4 T cells. However, only conventional DCs drove OT-II T cell proliferation and Th2 cell differentiation. After diphtheria toxin administration both monocyte–derived and conventional DCs were ablated and OT-II T cell priming and differentiation no longer occurred (Kool et al., 2008). During parasite infection with *Schistosoma mansoni* (Phythian-Adams et al., 2010) or *Nippostrongylus brasiliensis* (Smith et al., 2011), $CD11c^+$ cells also play an important role in driving type 2 immune responses as their ablation resulted in dramatically impaired Th2 responses and a marked shift towards inflammatory IFN-γ production. Thus, $CD11c^+$ cells, in particular conventional DCs, are necessary to drive Th2 cell differentiation in allergic, adjuvant-driven and parasitic conditions.

The first indication that certain subsets of DCs may be specialized to induce Th2 responses *in vivo* came from a study that transferred antigen-pulsed splenic DCs into the footpad of recipient animals and assessed their potential to drive T cell responses. It was observed that transferred $CD8^+$ DCs induced antigen-specific IL-2 and IFN-γ secretion, whereas $CD8^-$ DCs preferentially induced IL-4, IL-5 and

IL-10. Hereby, transferred DCs induced acute responses as well as memory responses, measured 14 days after immunization. IFN-γ responses were dependent on the ability of DCs to produce IL-12, as transfers of IL-12$^{-/-}$ CD8^{+} DCs demonstrated a reduced potential to induce IFN-γ responses, whereas Th2 induction by IL-12$^{-/-}$ CD8^{-} DCs remained unaffected (Maldonado-López et al., 1999). A follow-up study by the same group assessed the necessity of further cytokines to be produced by DCs to induce the observed immune responses. Apart from IL-12, IFN-γ production by CD8^{+} DCs was important to drive antigen specific Th1 responses in the recipient animals, while IL-12$^{-/-}$ and IFN-γ$^{-/-}$ CD8^{-} DCs induced similar Th2 response as their wild-type counterparts. Interestingly, IL-4 did not seem to be necessary for the induction of the Th2 response, as IL-4$^{-/-}$ DCs induced similar Th2 responses as their wild-type counterparts. However, the transfer of IL-10 deficient CD8^{-} DCs showed lower Th2 responses, suggesting that IL-10 production by DCs is required for optimal Th2 development (Maldonado-López et al., 2001). Similar results were observed when BMDCs from different transgenic mouse strains were pulsed with antigen and transferred into the footpad of wild type recipient mice. Heat-killed *Propionibacterium acnes* (Pa) was hereby used for a Th1 and SEA for a Th2 stimulus. As expected, SEA-pulsed BMDCs induced Th2 responses, measured by IL-4, IL-5 and IL-13 secretion in restimulation cultures, whereas Pa pulsed BMDCs induced strong IFN-γ responses. SEA-specific Th2 and Pa-specific Th1 responses were unaffected if antigen-pulsed IL-4$^{-/-}$ BMDCs were transferred. However, Pa-specific Th1 responses were reduced after the transfer of IL-12$^{-/-}$ BMDCs, whereas SEA specific Th2 responses were not affected (MacDonald et al., 2002). Taken together these data suggest that Th2 differentiation is not a default response in the absence of Th1-polarizing signalling but requires specific signals from DCs.

1.4.2 Signalling pathways by which dendritic cells drive Th2 immune responses

Several mechanisms have been proposed by which dendritic cells influence Th2 responses. However, it is important to realize that different Th2 inducing stimuli can activate different signalling pathways. The signalling pathways presented here represent some of the better understood model mechanisms but are neither conclusive nor parasite specific. Furthermore, most studies have taken advantage

of *in vitro* differentiated bone marrow dendritic cells (BMDCs), which only have little similarity to tissue-derived DCs, and contain heterogeneous populations of monocytes, macrophages, monocyte–derived and conventional DCs, which all respond differently to the presented stimuli (Helft et al., 2015).

One of the early candidates that was suspected to be produced by DCs to drive Th2 differentiation was IL-4. Although IL-4 is required to differentiate Th2 cells *in vitro* and IL-4$^{-/-}$ and IL-4R$^{-/-}$ mice cannot mount Th2 responses, it is not produced by DCs and IL-4$^{-/-}$ DCs induce regular Th2 responses (MacDonald and Pearce, 2002; Maldonado-López et al., 2001). It has been suggested that other cells, like basophils, eosinophils or ILC2s exogenously provide the required IL-4 for the initial step of Th2 differentiation, but it has also been demonstrated that naive CD4 T cells can produce IL-4 in low amounts upon TCR activation *in vitro* (Yamane et al., 2005). However, other cytokines have been associated with the ability of DCs to drive Th2 differentiation. One of these cytokines is IL-10, and IL-10-deficient DCs have been shown to drive less potent Th2 responses. For example, the transfer of antigen-pulsed IL-10$^{-/-}$ splenic CD8^{-} DCs induced lower Th2 responses compared to the transfer of control DCs. Furthermore, IL-12p40 production was increased in these DCs, leading to increased IFN-γ responses after transfer. Incubation with exogenous IL-10 inhibited the secretion of IL-12p40, suggesting that IL-10 regulates Th1 induction rather than directly induce Th2 responses (Maldonado-López et al., 2001). In accordance, several studies have demonstrated that DCs do not undergo conventional maturation when incubated with parasite antigen. For instance, in contrast to BMDCs incubated with *Propionebacterium acnes*, SEA-exposed BMDCs did not upregulate classical costimulatory markers like CD40, CD80 or CD86 and did not produce IL-4, IL-10 or IL-12. Furthermore, no phenotypical difference in regards to the expression of the costimulatory markers CD40, CD80 and CD86 was observed between CD8^{-} splenic DCs harvested from wild type or *S. mansoni*-infected animals (MacDonald et al., 2001). Similar observations were made in human monocyte-derived dendritic cells, which did not upregulate CD80, CD83 or CD86, nor produced TNF-α, IL-6 or IL-12 after incubation with SEA. Furthermore, Toll-like receptor induced maturation of human monocyte-derived DCs with poly-I:C or LPS was reduced by SEA, downregulating the expression of CD80, CD83 and CD86 and the production of TNF-α, IL-6 and IL-12 (van Liempt et al., 2007). Interestingly, this effect was completely reproduced by incubating murine or human derived DCs with the *S. mansoni* egg antigen

omega-1, which also inhibited LPS-induced IL-12 secretion and CD86 expression (Everts et al., 2009). This suggests that parasite antigens activate cellular pathways in DCs that promote Th2 differentiation while inhibiting proinflammatory signals and producing IL-10 (Fig. 1-9).

Another mechanism proposed to drive DCs to induce Th2 responses, is via thymic stromal lymphopoietin (TSLP) signalling. TSLP is produced by epithelial cells at barrier surfaces, such as the skin, lung and intestine and has been shown to promote pathogenic Th2 responses, such as atopic dermatitis and asthma (Ziegler and Artis, 2010). TSLP-stimulated human blood DCs responded by upregulating the costimulatory markers CD40, CD80 and CD86 and producing the Th2-attracting chemokine TARC/CCL17. However, no secretion of the proinflammatory cytokines IL-1β, IL-6, IL-12 and TNF-α was detected. *In vitro* cocultures with CD4 T cells revealed that TSLP-stimulated DCs induced Th2 cell differentiation, which secreted IL-4, IL-5 and IL-13 as well as TNF-α and low amounts of IFN-γ (Soumelis et al., 2002). In mice, TSLP expression was increased in the lungs of mice with allergen-induced asthma and TSLP receptor (TSLPR)-deficient mice showed decreased levels of disease. Similar to human DCs, BMDCs upregulated CD80 and CD86 during *in vitro* incubation with TSLP and produced CCL17 (Zhou et al., 2005). A proposed signalling pathway for TSLP is the binding to signal transducer and activator of transcription 5 (STAT5). STAT5 expression is dispensable for DC homeostasis and function but required for TSLP-dependent Th2 response-mediated disorders, such as antigen-induced airway inflammation and contact hypersensitivity. Flt3L-generated $CD11b^{+}$ BMDCs from $Stat5^{f/f}$ CD11c-cre-positive mice showed no upregulation of CD80, CD86 and OX40L nor CCL17 production in response to TSLP stimulation and were not able to drive TSLP-potentiated Th2 differentiation *in vitro* (Bell et al., 2013). Further signalling pathways by which TSLP acts on DCs were identified by Arima *et al*. They identified that the activation of the nuclear factor kappa B (NF-κB) subunits p50 and RelB by TSLP led to the upregulation of OX40L and that the activation of STAT6 triggered the secretion of CCL17. In addition, TSLP signalling limited the activation of STAT4 and IRF8, which are essential for the production of IL-12. In contrast, LPS did not activate STAT6, but instead increased the abundance of STAT4 and IRF8, which led to the production of IL-12 in BMDCs (Arima et al., 2010). Thus, TSLP mainly influences immune responses by blocking proinflammatory signalling in DCs that lead to Th1 responses and drives the

upregulation of OX40L and the secretion of CCL17, which have been associated with promoting Th2 responses.

In addition to pathogenic Th2 responses, like allergy, asthma or atopic dermatitis, TSLP-TSLPR interactions also influence host protective immunity after the exposure to helminth parasites. Administration of monoclonal antibodies to TSLP or deletion of TSLPR in *Trichuris muris*-infected animals resulted in defective Th2 responses, elevated production of IFN-γ and IL-17 and a failure to control infection. As previously observed, TSLP was necessary to control IL-12 production in DCs and $TSLPR^{-/-}$ splenic DCs from uninfected or infected animals produced more IL-12 than their wild type counterparts (Taylor et al., 2009). However, TSLP-TSLPR interactions are not necessary for the induction of Th2 responses in all parasitic infections. For example, TSLPR signalling was dispensable for Th2 responses in *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* infections. The authors demonstrated that excreted or secreted antigens from these parasites, but not from *T. muris*, could directly limit the expression of IL-12 by BMDCs, making the function of TSLP to suppress IL-12 secretion redundant (Massacand et al., 2009). Similarly, *S. mansoni* infected $TSLPR^{-/-}$ mice showed reduced IL-5 and IL-13 responses during acute (9 week) and chronic (12 week) infection but the general course of the disease and physiology of the infected mice, such as lesion formation and granuloma size, were not affected (Ramalingam et al., 2009). Thus, TSLP signalling plays a major role in pathogenic Th2 responses, by blocking proinflammatory stimuli in DCs, but is dispensable in many parasite infections.

Besides the inhibition of IL-12 production by TSLP or helminth-derived molecules, direct activators of Th2 responses also play an important role in DC-driven type 2 immunity. Maturation of human monocyte-derived dendritic cells with TSLP induced the expression of costimulatory molecules such as CD80 and CD86 and the secretion of CCL17, but furthermore led to the expression of OX40L, IRF4 and Jagged1. Blocking of OX40L using monoclonal antibodies led to impaired Th2 differentiation *in vitro*, and OX40-OX40L Th2 differentiation was inhibited by providing exogenous IL-12 (Ito et al., 2005). Thus, OX40-OX40L signalling is required for the differentiation of Th2 cells but requires the inhibition of IL-12 to induce efficient Th2 responses.

Similar observations were made in human monocyte-derived dendritic cells incubated with SEA or cholera toxin, which drove Th2 responses *in vitro* and expressed OX40L, while their production of IL-12 was inhibited (de Jong et al., 2002). Treating mice with OX40L-blocking antibodies substantially inhibited TSLP-induced allergic inflammation in the lung and skin, including Th2 cell infiltration, cytokine secretion and IgE production and inhibited antigen-driven asthma in mice and rhesus monkeys (Seshasayee et al., 2007). Similarly, murine SEA-pulsed $OX40L^{-/-}$ BMDCs could not induce antigen-specific Th2 responses when transferred into naive recipient animals, due to ineffective Th2 cell expansion in these mice. Th2 responses could hereby be rescued by the injection of agonistic anti-OX40 antibodies, indicating that OX40L expression by DCs was important to drive appropriate Th2 responses (Jenkins et al., 2007). Further evidence that OX40-OX40L interactions directly influence Th2 differentiation of naive T cells was provided by Flynn *et al*. They demonstrated that *in vitro* stimulation of naive CD4 T cells through OX40L promoted IL-4 expression, and inhibited IFN-γ production in CD8 T cells and IL-12–stimulated CD4 T cells (Flynn et al., 1998).

Thus, OX40L expression by DCs, which can be induced by TSLP or SEA, is an important factor that directly influences Th2 differentiation (Fig. 1-9). Apart from costimulatory molecules such as OX40L and cytokines like IL-10, transcription factors like IRF4 have been implicated in type 2 immunity and the experiments supporting the role of IRF4 in driving Th2 immune responses are discussed in the following section.

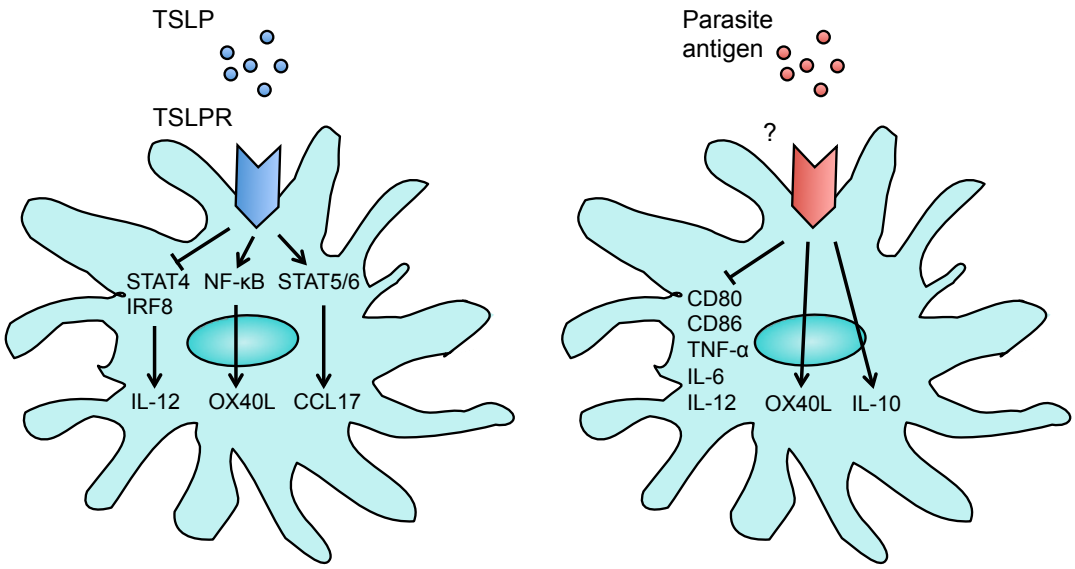

Figure 1-9. Both TSLP and parasite antigen limit proinflammatory signalling and upregulate OX40L expression in DCs

TSLP signals through the TSLP receptor (TSLPR) and upregulates the expression of OX40L through the activation of NF-κB and CCL17 through STAT5/6, while blocking STAT4 and IRF8, which are important in IL-12 production. Parasite antigens, such as SEA, also limit proinflammatory signalling and inhibit the upregulation of the costimulatory molecules CD80 and CD86 and the production of TNF-α, IL-6 and IL-12, while promoting OX40L expression and IL-10 production. However, the cellular receptors and signalling mechanisms controlling the effects of these parasite antigens on DCs are less well understood.

1.4.3 The role of IRF4 in type 2 immunity

IRF4 is an important regulator of type 2 immunity and is expressed in many cell types. It is a member of the interferon-regulatory family of transcription factors and is expressed by Th2 cells and critical for their differentiation and function. In the absence of IRF4, IL-4 fails to induce Th2 cell differentiation *in vitro*, and Th2 responses are absent in *Leishmania major* infected IRF4$^{-/-}$ animals. Although early IL-4 signalling is normal in IRF4$^{-/-}$ T cells, GATA-3 is not upregulated, leading to impaired Th2 differentiation (Lohoff et al., 2002). Furthermore, the expression of IRF4 is also important for the ability of Foxp3^{+} regulatory T cells to specifically inhibit Th2 responses and the specific deletion of IRF4 in Foxp3^{+} cells leads to type 2 mediated autoimmune disease (Zheng et al., 2009). IRF4 also controls plasma cell differentiation and class switching, and conditional deletion of IRF4 in germinal center B cells blocks their differentiation into post-germinal center plasma cells (Klein et al., 2006).

Apart from T and B cells, IRF4 also plays an important role in antigen presenting cells. It is a key regulator for the alternative activation of macrophages, which are induced during type 2 immune responses (Gordon, 2003), and the induction of genes associated with alternative activation is severely impaired in IRF4$^{-/-}$ bone marrow macrophages or in macrophages harvested from IRF4$^{-/-}$ mice after chitin administration (Satoh et al., 2010). IRF4 has also been shown to interact with MyD88 and acts as a negative regulator of TLR signalling. TLR-dependent induction of the proinflammatory cytokines IL-6, IL-12 and TNF-α is markedly enhanced in IRF4$^{-/-}$ peritoneal macrophages *in vitro*, whereas this induction does not occur after ectopic expression of IRF4 in a macrophage cell line (Negishi et al., 2005).

The importance of IRF4 expression in dendritic cells has been demonstrated in multiple studies and developmental as well as functional roles have been identified. As previously described, IRF4 is expressed by CD11b^{+}CD8^{-} splenic DCs, CD11b^{+} lymph node DCs and CD11b-expressing DCs in peripheral tissues. In IRF4$^{-/-}$ mice CD11b^{+}CD8^{-} splenic DCs are significantly reduced in number and their development is severely impaired. Also CD11b^{+} BMDCs can not be generated from IRF4$^{-/-}$ bone marrow cells *in vitro* (Suzuki et al., 2004b). Furthermore, conditional deletion of IRF4 leads to decreased numbers of CD11b^{+}CD103^{+} DP DCs in the intestine (Persson et al., 2013b) and CD11b^{+}CD24^{+} DCs in the skin (Schlitzer et al., 2013) and impaired survival of these subsets has been suggested as a potential mechanism. However, IRF4 also acts on the migration of CD11b^{+} DCs in the skin. In steady state the numbers of migratory Langerhans cells and CD11b^{+} DCs were reduced in the cutaneous lymph nodes of IRF4$^{-/-}$ mice. Furthermore, upon induction of skin inflammation, IRF4$^{-/-}$ CD11b^{+} DCs failed to upregulate CCR7 and did not migrate to the cutaneous lymph nodes, whereas the migration of Langerhans cells and CD103^{+} DCs was not affected (Bajaña et al., 2012). Similarly, fewer CD11b^{+}CD103^{-} and CD11b^{+}CD103^{+} DP migratory DCs were observed in the MLNs of steady-state IRF4$^{f/f}$ CD11c-cre-positive mice (Persson et al., 2013b). Genetic deletion of IRF4 in CD11c^{+} cells also led to the absence of PDL2^{hi} CD301b^{+} migratory DCs from the skin-draining lymph nodes but not from the skin itself. Th2 cell-mediated responses in these animals were reduced in response to immunization with the protease adjuvant papain and after *N. brasiliensis* infection (Gao et al., 2013). Thus, apart from developmental and migratory effects of IRF4 on CD11b^{+} DCs, IRF4 also plays an important direct functional role in driving Th2 responses.

One proposed mechanism is the impaired formation of peptide-MHC class II complexes by IRF4$^{f/f}$ CD11c-cre-positive CD11b^{+} DCs, as observed in splenic DCs and BMDCs. This deficiency resulted in reduced CD4 T cell proliferation in *in vitro* cocultures and impaired helper T cell but not cytotoxic T lymphocyte responses during allergic airway inflammation. Further analyses in BMDCs identified an IRF4-dependent regulatory module that specifically drives peptide-MHC class II complex formation through the genes *H2-DMb2*, *Ctss* and *Cst3* (Vander Lugt et al., 2014). IRF4 also directly targets and activates the *Il10* and *Il33* genes in DCs. Using a late expressed construct of CD11c-cre the authors were able to circumvent the developmental effects of IRF4 deletion on DCs and focus on its functional role in Th2 immunity. Similar to previous observations, IRF4-expression in DCs was necessary for the development of Th2-mediated house dust mite (HDM) allergic airway inflammation, but not required for the development of pulmonary Th1 antiviral responses. IRF4-deficient BMDCs failed to prime Th2 cells *in vitro*, while Th1 and Th17 priming was unaffected. Specifically, IRF4 promoted Th2 differentiation through the upregulation of IL-10 and IL-33 in BMDCs, and the administration of exogenous IL-10 and IL-33 recovered the ability of IRF4-deficient DCs to promote Th2 differentiation *in vitro*. Furthermore, it was observed that IRF4 and its binding partner PU.1 directly bind to the *Il10* and *Il33* gene locus (Williams et al., 2013). Apart from IL-10, which is involved in regulating inflammatory responses, IL-33 is an important mediator that drives allergic responses and has been shown to play an important role in DC-driven HDM induced atopic asthma. Incubation with HDM *in vitro* induced the expression of IL-33 in BMDCs, which was dependent on FcRγ and TLR4. FcRγ-deficient mice did not develop atopic asthma in response to HDM, but exogenous IL-33 was sufficient to restore pathogenesis and the development of Th2 responses.

The critical role for DCs to drive this response was shown by adoptive transfer of allergen-pulsed BMDCs. The transfer of wild type BMDCs was sufficient to induce allergic responses and Th2 development in FcRγ$^{-/-}$ mice through the production of IL-33, whereas FcRγ-deficient BMDCs did not induce such responses (Tjota et al., 2014). It has also been shown that the binding partner of IRF4, PU.1 directly binds to the OX40L promoter in BMDCs and human monocyte-derived DCs and that PU.1 knockdown decreased the expression of OX40L in stimulated BMDCs (Yashiro et al., 2016).

However, most of these insights were identified by using *in vitro* generated BMDCs and it remains to be determined whether the same mechanisms apply to tissue derived DCs *in vivo*. Therefore, one of the aims of this project is to analyse the effects of IRF4 deletion on intestinal DCs using IRF4$^{f/f}$ CD11c-cre mice and to determine whether they are less able to induce Th2 responses against *S. mansoni* egg antigens.

1.5 Hypothesis and aims

In order to investigate the induction of Th2 responses by *S. mansoni* eggs our first aim was to develop an immunization protocol in the intestine. *S. mansoni* eggs have been shown to drive potent Th2 responses. Inflammatory responses are predominant in the first 6-8 weeks of infection. Only upon the production of eggs by adult female worms does the immune response shift towards type 2 immunity. However, the exact timing and dose of egg production can not be controlled during live infection, making the specific study of the induction of Th2 responses against *S. mansoni* eggs impossible during live infection. It has been demonstrated that the injection of non-viable freeze-thawed eggs is sufficient to drive Th2 responses in the footpad and we adapted this procedure for the intestine (MacDonald et al., 2002). In order to represent eggs that penetrate the intestinal wall, where they cause damage, generate granuloma formation and drive Th2 responses during chronic schistosomiasis, we deposited a defined number of eggs into the intestinal wall and assessed the immune responses they induced locally and in the draining lymph nodes (see chapter 3).

An important regulator of type 2 immunity is the transcription factor IRF4, which controls the development of Th2 cells, post-germinal center plasma cells, alternatively activated macrophages and CD11b-expressing dendritic cells (DCs), which all play a role in type 2 immunity. In the case of DCs IRF4 is not only involved in their development but has also been shown to play a role in cell migration and directly regulating BMDC-driven Th2 differentiation via peptide-MHC class II complex formation (Vander Lugt et al., 2014) and the expression of IL-10, IL-33 and OX40L (Williams et al., 2013). However, these effects have not been studied in the context of intestinal Th2 responses. Thus, our second aim was to analyse the effects of IRF4 deletion on intestinal CD11c-expressing cells *in vivo*

using IRF4$^{f/f}$ CD11c-cre mice. We determined whether this IRF4 deletion caused impaired Th2 responses against *S. mansoni* egg antigens and which CD11c-expressing cells were affected. We hereby focused on cells that took up egg antigens in the intestine and transported them to the draining lymph nodes to be presented to T cells (see chapter 4).

As DCs are specialized to sample intestinal antigens, migrate to the draining lymph nodes and present and prime antigen specific T cells, we hypothesised that DCs are instrumental in this process. The necessity of CD11c^{+} cells to drive type 2 immune responses during *S. mansoni* infection has been demonstrated using CD11c-DTR mice (Phythian-Adams et al., 2010). However, it remains unclear whether these cells are important for the induction, maintenance or restimulation of the response. Furthermore, CD11c is expressed by various antigen presenting cells, including macrophages and DCs. Therefore, our third aim was to determine which of these CD11c-expressing cell populations was involved in driving Th2 responses against *S. mansoni* eggs in the intestine and were sufficient to prime antigen specific Th2 cells in the draining lymph nodes.
The involvement of DCs in driving Th2 responses has been demonstrated in multiple model systems. However, the mechanisms by which DCs induce these responses are not well understood. Our limited understanding comes from *in vitro* generated BMDCs incubated with soluble egg antigen (SEA) *in vitro.* These cells showed limited upregulation of the costimulatory markers CD40, CD80 and CD86 in response to SEA (MacDonald et al., 2001), but required OX40L to induce effective Th2 responses (Jenkins et al., 2007). However, *in vitro* generated BMDCs contain heterogeneous populations of monocytes, macrophages, monocyte–derived and conventional DCs, which can all respond differently to the presented stimuli. Thus, we further aimed to investigate the effects of *S. mansoni* egg antigens on tissue-derived intestinal DCs to understand the molecular mechanisms that drive the differentiation of Th2 responses (see chapter 5).

Furthermore, the idea is emerging that specific DC subsets are specialized to perform different roles in T helper cell differentiation. This has been demonstrated for Th1, Th17 and Treg cells in the intestines but it is unclear if it also applies to the induction of Th2 cells. Therefore, the final aim of this project was to determine if specific intestinal DC subsets are specialized to prime Th2 cells and drive Th2 responses against *S. mansoni* egg antigens. It has been observed that the

composition of DC subsets varies between different regions of the gastrointestinal tract, with the most striking differences observed between the small intestine and colon (Denning et al., 2011). The small intestine and colon can be infected by numerous helminth parasites, some of which selectively affect the small intestine or colon (Grencis, 2015). We therefore defined the role of DC subsets that drive intestinal Th2 responses in a tissue-specific context and separately assessed the involvement of lamina propria, lymph migrating and lymph node DC subsets, from the small intestine and the colon (see chapter 6).

Chapter 2: Materials and Methods

2.1 Animals

C57BL/6 mice were ordered from Harlan UK at six weeks of age, whereas all other mouse strains were bred in house. C57BL/6.SJL (kindly provided by Prof. Allan Mowat from the University of Glasgow) and KN2 mice (kindly provided by Prof. Andrew MacDonald for the University of Manchester) were housed under specific pathogen free conditions at the Central Research Facility of the University of Glasgow. In general mice were housed in conventional cages. However, OT-II mice (kindly provided by Prof. Allan Mowat from the University of Glasgow) and animals that had undergone surgery were housed in individual ventilated cages. Age and gender matched adult animals were used in each individual experiment, which were approved by the local ethic committees, and performed under licenses issued by the UK Home Office. $IRF4^{f/f}$ CD11c-cre-positive and $IRF4^{f/f}$ cre-negative bone marrow chimeras were created by lethally irradiating 6-week old C57BL/6.SJL recipients with 10 Greys of irradiation and reconstituting them by intravenous injection with 2-4 x 10^6 $IRF4^{f/f}$ CD11c-cre-positive or $IRF4^{f/f}$ cre-negative bone marrow cells, which we received from our collaborators Dr. Marcus Svensson-Frej and Prof. William Agace from the University of Lund, Sweden. Chimeras were housed in individual ventilated cages and animals were monitored daily for 3 weeks to ensure animal well-being. Cell chimerism was assessed in venous blood 3 weeks after irradiation.

2.2 Surgical procedures

All surgical procedures were carried out under aseptic conditions and inhalation anaesthesia with Isoflurane (Abbot Animal Health). Before surgery, mice were shaved and treated with the subcutaneous analgesics Carprofen at a dose of 0.1 ml/100 g (Rimadyl; Pfizer) and Buprenorphine at 0.15 ml/100 g (Vetergesic; Reckitt Benckiser Healthcare).

For intestinal injections the intestines were exposed by midline incision and 1,000 non-viable freeze-thawed *S. mansoni* eggs (van der Werf et al., 2003), resuspended in 20 µl DPBS (Gibco); or 20 µl of DPBS or soluble antigen were

injected into the intestinal lamina propria of anaesthetized mice using a Micro-Fine Plus Hypodermic Syringe (29G x 12.7mm; BD). Prof. Andrew MacDonald from the University of Manchester kindly provided *S. mansoni* eggs that had been isolated under sterile conditions from the livers of infected C57BL/6 mice prior to cryopreservation (MacDonald et al., 2002). After injection the intestines were replaced into the body cavity, the muscle layer was sutured with 6.0 vicryl absorbable sutures (Johnson and Johnson) and the skin closed using surgical clips (Autoclip Wound Clip System, Harvard Apparatus).
Similarly, subcapsular injections were carried out on anaesthetized mice that had been fed 0.2 ml olive oil to visualize the MLN capsule 30 minutes prior to surgery. Intestines and MLNs were exposed and cells resuspended in 5 µl DPBS were injected under the MLN subcapsule using a Micro-Fine Plus Hypodermic Syringe (29G x 12.7mm; BD).

Mesenteric lymphadenectomy and thoracic duct cannulation procedures were carried out as previously described (Cerovic et al., 2015). For mesenteric lymphadenectomy an abdominal midline incision was made in 6-week-old male mice and the intestine exposed. For MLNx, all visible MLNs located above the superior mesenteric vein were removed by blunt dissection. For partial MLNx, the specific lymph nodes that drain the small intestine (sMLNx) or the colon (cMLNx) were removed (Houston et al., 2016). Upon completion the intestine was placed back into the body cavity, the muscle layer was sutured and the skin closed using surgical clips.
Six weeks after the MLNx procedure, mice were fed 0.2 ml olive oil, to visualize the lymphatics, 30 minutes prior to the surgical procedure. An incision of around 2-3 cm was made underneath the rib cage and a polyurethane medical grade intravascular tube (2Fr; Linton Instrumentation) was inserted into the thoracic duct, fixed in place using surgical glue, exteriorized between the ears and fed into a Covance harness (Harvard Apparatus). The muscle layer was then sutured, and the skin closed using surgical glue. Pseudo-afferent lymph was then collected for 18 hours on ice in DPBS supplemented with 20 U/ml of heparin sodium (Wockhardt).

2.3 Reagents

Complete RPMI contained RPMI 1640 medium supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol (all Gibco). FACS buffer consisted of DPBS supplemented with 2 mM EDTA and 1% FCS (all Gibco).

2.4 Cell isolations

Lymph collected overnight was passed through a 40 µm cell strainer (Greiner), and red blood cells lysed with 3 ml ammonium chloride potassium lysis buffer (Sigma Aldrich) for 1 minute and washed twice in complete RPMI.

MLNs were enzymatically digested as previously described (Houston et al., 2016) using RPMI (Gibco) supplemented with 8 U/ml Liberase and 10 µg/ml DNase (Sigma-Aldrich). MLNs were cut into small pieces and digested for 45 minutes at 37°C in a shaking incubator. To obtain single cell suspensions cells were passed through a 40 µm cell strainer (Greiner).

Intestinal digests were performed as described (Scott et al., 2016), with small intestines being digested in RPMI supplemented with 1 mg/ml Collagenase VIII (Sigma-Aldrich) and 10% FCS. Small intestines were excised and washed in Hanks' Balanced Salt Solution (HBSS, Gibco) supplemented with 2% FCS. Peyer's Patches and fat were removed and the intestine cut longitudinally and into 0.5 cm sections. Tissue was shaken vigorously in HBSS/2% FCS and filtered through a 50 µm Nitex mesh (Cadisch and Sons). Tissues were washed twice in HBSS supplemented with 2 mM EDTA at 37°C for 20 minutes, and then digested for 15 minutes at 37°C in a shaking incubator. Cells were subsequently passed through a 100 µm and a 40 µm cell strainer (BD Biosciences) and washed twice in complete RPMI. Similarly, colons were harvested and after removing fat and faeces, the colon was opened longitudinally and cut into 0.5 cm segments. After being filtered through a 50 µm Nitex mesh (Cadisch and Sons) and washed in HBSS/EDTA, colons were digested in RPMI supplemented with 0.425 mg/ml Collagenase V (Sigma-Aldrich), 0.425 mg/ml Collagenase D (Roche), 1 mg/ml Dispase (Gibco), 30 µg/ml DNase (Roche) and 10% FCS for 30-40 minutes at 37°C in a shaking incubator. Then cells were passed through a 40 µm cell strainer (BD Biosciences) and washed twice in complete RPMI.

2.5 Flow cytometry and cell sorting

Mouse tissue cell surface markers and intracellular cytokines were stained using combinations of fluorescently labelled primary antibodies at a standard dilution of 1:200. These included anti-CD4 (GK1.5 and RM4-5), anti-CD8a (53-6.7), anti-CD44 (IM7), anti-CD45R/B220 (RA3-6B2), anti-CD11c (N418), anti-I-A/I-E (M5/114.15.2), anti-CD11b (M1/70), anti-CD103 (2E7), anti-CD64 (X54-5/7.1), anti-Ly6C (HK1.4), anti-TCR Vα2 (B20.1), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104) and anti-human CD2 (RPA-2.10) purchased from Biolegend, and IFN-γ (XMG1.2) and anti-IL13 (eBio13A) from eBioscience. SEA was fluorescently labelled using the Microscale Antibody Labelling Kit (Life Technologies) as instructed. Cell surface staining was performed on freshly isolated cells resuspended in FACS buffer and samples were stained in 12x75 mm polystyrene tubes (BD Biosciences) with up to 10 x 10^6 cells per 200 µl of FACS buffer. Non-specific binding of antibodies to Fc receptors was blocked by the addition of Fc block (anti-CD16/CD32, Biolegend) diluted 1:200 for 10 minutes at 4°C. Required antibodies or isotype controls were then added to samples for 30 minutes at 4°C and 5 µl 7AAD (Biolegend) or Fixable Viability Dye eFluor780 (eBioscience) diluted at 1:1000 were added to exclude dead cells from analysis. After staining, cells were washed twice in FACS buffer and analysed using a LSRII flow cytometer running FACSDiva Software (BD Bioscience) and analysed using Flowjo Software (Tree Star).

For cell sorting, cells were stained under sterile conditions and DCs were gated on by selecting live $CD45^+$ $CD45R/B220^-$ $CD64^-$ $Ly6C^-$ $CD11c^{hi}$ $MHCII^{hi}$ single cells and CD11b/CD103 expressing subsets sorted separately using an AriaIII cell sorter (BD Bioscience). Cells were collected in complete RPMI on ice and, for subsequent antigen loading, were incubated in supplemented (as above) X-vivo 15 media (Lonza) with 15 µg/ml SEA for 6 hours or 2 mg/ml OVA protein (Sigma-Aldrich) for 2 hours at 37°C and 5% CO_2.

2.6 *In vitro* cell cultures

For restimulation cultures 1 x 10^6 MLN cells were cultured in X-vivo 15 media (Lonza) supplemented with 1% L-glutamine (Invitrogen), 0.1% 2-mercaptoethanol

(Sigma-Aldrich) and 15 µg/ml SEA in round bottom 96-well plates (Corning) at 37°C and 5% CO_2. SEA was kindly provided by Prof. Andrew MacDonald from the University of Manchester and prepared as previously described (MacDonald et al., 2002). Supernatants were collected after three days and cytokines detected using the IL-4, IL-5, IL-13 and IFN-γ "ready-set-go" ELISA kits (eBioscience) following the manufacturer's instructions.

For intracellular staining experiments 2-4 x 10^6 MLN cells were incubated in RPMI supplemented with 2.5 ng/ml PMA (Sigma-Aldrich), 1 µg/ml ionomycin (Invitrogen), 0.5% GolgiStop (BD Bioscience) and 10% FCS for 4 hours at 37°C after which cell surface markers were stained. Alternatively, cells were incubated in flat-bottom 96 well plates (Corning) with 5 µg/ml plate-bound anti-CD3 and 2 µg/ml soluble anti-CD28 for 18 hours at 37°C and 5% CO_2. Subsequently cells were fixed and permeabilized using the eBioscience Foxp3/Transcription Factor Staining Buffer Set and intracellular staining was performed following the manufacturer's instructions.

For *in vitro* OT-II cocultures 2 x 10^5 OT-II MLN cells were labelled with CFSE (eBioscience) diluted at 1:1000 for 1 minute in DPBS, washed twice with complete RPMI and cocultured with 6,000 sMLN or 3,000 cMLN FACS-sorted DCs from each subset. Each subset had been pulsed with 2 mg/ml of OVA (Worthington, Lakewood) for 2 hours at 37°C and then extensively washed. After 3 days of coculture in round bottom 96 well plates (Corning) at 37°C and 5% CO_2, cells were stained for flow cytometry and CFSE dilution was assessed to measure OT-II T cell proliferation.

2.7 Statistical analysis

Prism Software (GraphPad) was used to calculate the SEM. Statistical differences between groups were calculated using Student's *t* tests, Mann-Whitney *U* tests and Kruskal-Wallis tests, were appropriate, with $P < 0.05$ being considered as significant. Non-parametric Mann-Whitney *U* tests were applied when samples were not normally distributed but followed a skewed distribution.

Chapter 3: Intestinal *Schistosoma mansoni* egg injections induce robust Th2 responses in the draining lymph nodes

3.1 Results

Type 2 immune responses are mounted against parasitic or allergic stimuli, that either protect against parasites, or exacerbate allergic conditions (Licona-Limón et al., 2013). The majority of parasites affect the gastrointestinal tract (Pulendran and Artis, 2012), many of which selectively affect the small intestine or colon (Grencis, 2015).

Schistosoma mansoni eggs, which penetrate the small intestine during live infection, are strong inducers of Th2 responses (Pearce and MacDonald, 2002). Intact live eggs, non-viable freeze-thawed eggs and their soluble egg antigens (SEA) all drive potent Th2 responses in both the parasitic infection (Pearce et al., 2004; Pearce and MacDonald, 2002) and in experimental models of egg or SEA injections (MacDonald et al., 2002; Klaver et al., 2015). Despite the facts that *S. mansoni* eggs predominately affect the small intestine, and that most parasites invade the gastrointestinal tract, little work has been done to specifically investigate the induction of the immune responses mounted against parasite antigens in the intestine.

Therefore, we aimed to establish an experimental immunization protocol using *S. mansoni* eggs in the intestine that would allow us to study the cellular mechanisms in the small intestine and colon that induce type 2 immune responses under controlled and physiologically relevant conditions.

3.1.1 Subserosal injection of *Schistosoma mansoni* eggs induces antigen specific immune responses in the MLNs

Non-viable freeze-thawed *S. mansoni* eggs and SEA were supplied by our collaborator Andrew MacDonald, who collected them from the livers of *S. mansoni* infected mice (MacDonald et al., 2002). In already published *S. mansoni* egg immunization models, eggs were injected into the footpad (Phythian-Adams et al., 2010), a tissue that is readily accessible. To deliver eggs into the gastrointestinal tract, we first considered the use of non-surgical procedures, like oral gavage and intrarectal administration. However, these methods were considered not to be suitable, as the number of eggs that would reach the small intestine or colon could not be precisely controlled using oral gavage, and intrarectal injections would not allow delivery of eggs into the small intestine. Furthermore, using these techniques eggs would be delivered into the intestinal lumen. However during live infection, eggs are located within the intestinal tissue as they penetrate the intestinal wall, the lamina propria and the epithelium to reach the intestinal lumen and be excreted.

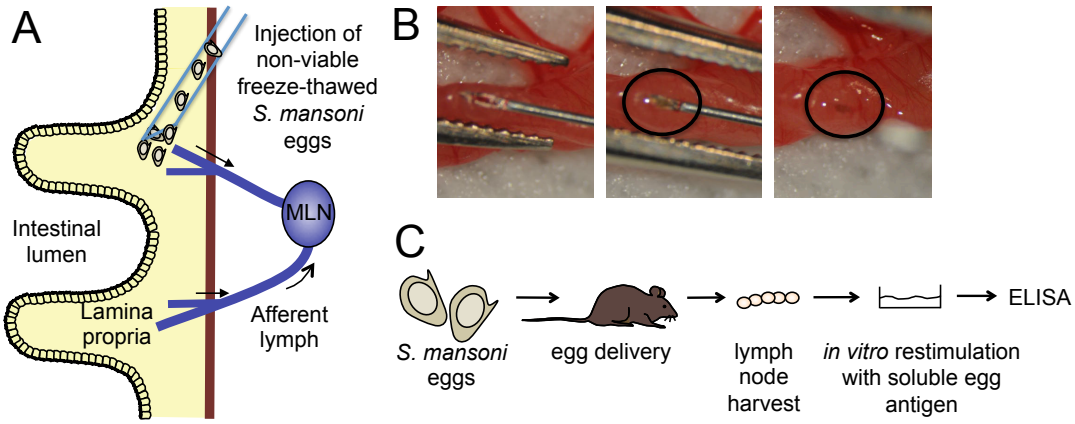

Figure 3-1. Injection of *S. mansoni* eggs into the intestinal lamina propria serves as an experimental model that represents penetrating eggs during live infection

Schematic illustrating the injection of *S. mansoni* eggs into the lamina propria of the small intestine (A). The cluster of injected eggs remained visible after injection, confirming that eggs were correctly delivered into the lamina propria and not the intestinal lumen (B). After immunization, lymph nodes were harvested and restimulated with soluble egg antigen *in vitro*. Released cytokines were detected by ELISA (C), revealing the magnitude of the induced immune response.

We therefore considered that injection into the intestine was potentially a suitable method for accurate delivery of eggs. The injection of eggs directly into the subserosal layer of the intestine was considered to be a relevant physiological location. During live infection *S. mansoni* eggs penetrate the intestinal wall to reach the intestinal lumen and be excreted. Some eggs become lodged within the intestine and induce strong Th2 responses, which after prolonged exposure lead to granuloma formation, a key hallmark of schistosomiasis (Wynn et al., 2004). The lamina propria is furthermore an important tissue for intestinal immune responses. Here, tissue resident antigen presenting cells (APCs), such as macrophages and migratory dendritic cells (DCs), detect and sample antigen. The APCs then communicate with T cells and other effector cells to mount appropriate immune responses, both within the tissue and the draining lymph nodes. As the intestinal lamina propria is drained by the afferent lymphatics that feed into the mesenteric lymph nodes (MLN) we monitored the induction of immune responses in the MLNs (Fig. 3-1 A). Here, DCs that migrate from the intestine to the draining lymph nodes present antigen to naive T cells and thus induce T cell responses.

To inject eggs under the serosal layer of the intestine, mice were anaesthetized, the skin and muscle layer were opened and the gastrointestinal tract was displayed. 1,000 non-viable *S. mansoni* eggs, resuspended in 20 µl of DPBS, were then injected into the subserosal layer (Fig. 3-1 B). The single injection site displayed no bleeding or other damage after injection and the injected cluster of eggs remained clearly visible and could even be located 5 days after the surgery. This demonstrated that the precise injection into the intestinal tissue caused minimal damage to the tissue, and simulated lodged eggs that remained at the site of injection for at least 5 days. After injection, the intestines were moved back into the abdominal cavity. The muscle layer and skin incisions were closed and the mouse was placed in a heat cabinet for recovery. After an immunization period of 5 days, the draining lymph nodes were harvested and the induction of antigen specific Th2 responses analysed. Initially these Th2 responses were assessed using restimulation cultures, as previously described in the literature (MacDonald et al., 2001; MacDonald and Pearce, 2002). 1 x 10^6 cells, obtained from lymph node cell suspensions, were cultured with soluble egg antigen *in vitro*. After 3 days supernatants were collected and the production of the cytokines IFN-γ, IL-4, IL-5 and IL-13 was measured by ELISA (Fig. 3-1 C).

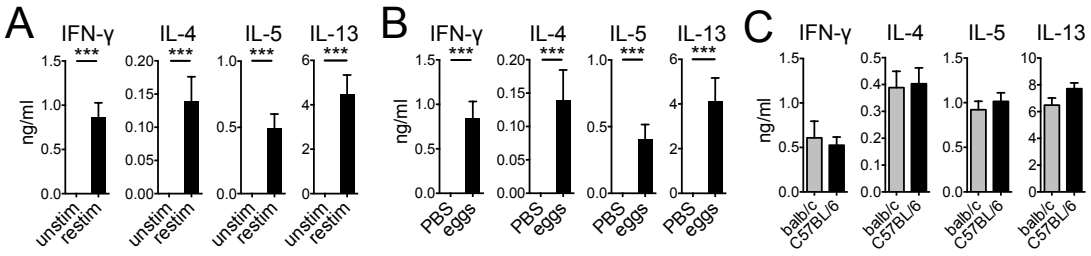

Figure 3-2. Intestinal egg injection induces antigen specific immune responses in the MLNs after restimulation with SEA

5 days after egg injection, 3-day restimulation cultures were prepared from MLN cells by culturing 1x10^6 MLN cells with 7.5 µg/ml SEA. The release of IFN-γ, IL-4, IL-5 and IL-13 was measured by ELISA in supernatants from cells that were not restimulated (unstim) or were restimulated (restim) with SEA (A). Cytokine responses were also measured after SEA restimulation of PBS or egg injected mice, 5 days after injection (B). Responses after SEA restimulation of MLN cells harvested 5 days after egg injection into BALB/c or C57BL/6 mice (C). Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (***P ≤ 0.001).

By using restimulation cultures we could measure an antigen specific immune response that developed in the draining lymph node during the immunization period. Responses to the restimulating agent, in our case soluble egg antigen (SEA) derived from *S. mansoni* eggs, could be detected by measuring cytokines released into the supernatant. The level of produced cytokines also indicated the strength of the induced immune response. By analysing different key cytokines we were able to classify the nature of the immune response and measured IFN-γ, which is an inflammatory cytokine that is also induced by *S. mansoni* egg antigens (Cook et al., 2015), and IL-4, IL-5 and IL-13, which are associated with type 2 immune responses and produced by Th2 cells (Constant and Bottomly, 1997).

In an initial experiment MLNs were harvested 5 days after subserosal egg injection. After 3 days of *in vitro* culture the supernatants of unstimulated and restimulated MLN cells were analysed. We could detect IFN-γ as well as IL-4, IL-5 and IL-13 in the wells that were restimulated with SEA but none of these cytokines were detected in unstimulated wells (Fig. 3-2 A). IL-4, IL-5 and IL-13 were present at different concentrations after restimulation as they are produced by Th2 cells in different amounts (Paul, 2010). IL-4 was detected with an average concentration of 0.15 ng/ml, whereas the levels of IL-5 and IL-13 were 0.5 ng/ml and 4 ng/ml on average. The production of IFN-γ, a Type 1 cytokine, was not surprising, as *S.*

mansoni eggs contain a complex pool of different antigens, some of which can induce Type 1 responses (Cheever et al., 2002). As we did not detect any of these cytokines in unstimulated cell cultures, these cytokines were only secreted upon antigen specific restimulation. We therefore concluded that the use of restimulation cultures allowed the detection of an antigen specific response induced after subserosal injection in the MLNs without detectable background expression of cytokines. We could also measure the strength of the induced immune response by evaluating the concentrations of cytokines that were produced.

To determine if the surgical procedure of subserosal injections affected the immune response we compared egg injected and PBS injected mice after 5 days of immunization and measured antigen specific cytokines of MLN cells 3 days after restimulation. We observed that no cytokines were detectable in the supernatants from PBS-injected control mice, whereas the level of cytokine measured in egg-injected mice was comparable to what we had previously observed (Fig. 3-2 B). We therefore determined that the injection of eggs into small intestinal tissue provided a robust method of delivery, which induced antigen specific immune responses, and that the injection itself did not stimulate any background expression of cytokines that could interfere with our analysis.

Different mouse strains have been shown to have intrinsic preferences towards eliciting different immune responses due to their genetic background. For example immune responses against *Trichuris muris* in C57BL/6 and BALB/c mice are characterized by the production of Th2 associated cytokines and the expulsion of worms while Th1-associated cytokines promote chronic infections in genetically susceptible AKR/J mice (Antignano et al., 2011; Cliffe and Grencis, 2004). Infections with *S. mansoni* demonstrated that the different genetic background of C57BL/6 and BALB/c mice did not impact resistance, measured by worm and egg burden. However, larger granuloma sizes were observed in the livers of infected BALB/c mice and significantly higher IL-4 and IL-13 cytokines were produced by splenic T cells after infection and reinfection, suggesting that BALB/c mice produced stronger Th2 responses against *S. mansoni* eggs (Alves et al., 2016). To address whether genetic host backgrounds would influence our intestinal *S. mansoni* egg immunization model we compared the immune responses induced by subserosal egg injection in C57BL/6 and BALB/c mice. Again, we injected 1,000 non-viable eggs under the serosal layer of the small intestine and harvested

the MLNs 5 days after egg injection. We observed that restimulated MLN cells from both mouse strains produced similar amounts of IFN-γ, IL-4, IL-5 and IL-13 and we could not detect any statistically significant difference between any of these cytokines (Fig. 3-2 C). This suggested that the intestinal injection of *S. mansoni* eggs induced a comparable immune response in the MLNs of C57BL/6 and BALB/c mice and that transgenic mice from both backgrounds could be used to further examine the underlying cellular processes involved in the induction of this response.

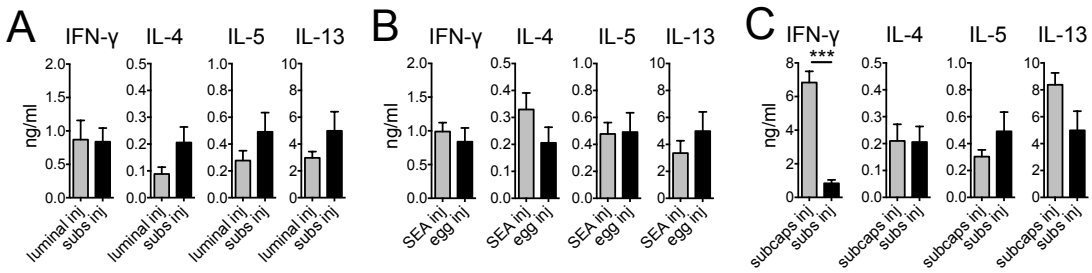

Figure 3-3. The intestinal wall represents a physiologically relevant location for intestinal egg injection

Cytokine responses were measured in restimulation cultures from MLN cells that were harvested 5 days after luminal (luminal inj) or subserosal (subs inj) egg injections into small intestines of mice (A). The release of IFN-γ, IL-4, IL-5 and IL-13 after 3 days of restimulation with SEA was compared between intestinal injections of 20 µg soluble egg antigen (SEA inj) or 1,000 non-viable eggs (egg inj) (B). Immune responses were measured after egg injection into the MLN subcapsular space (subcaps inj) or the intestinal subserosal tissue (subs inj) (C). MLNs were harvested after 5 days and restimulated with SEA for another 3 days. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (***P ≤ 0.001).

We had established that intestinal egg injections induced robust immune responses in the MLNs, which we detected using restimulation cultures. We proceeded by testing whether the injection of eggs into different layers of the intestine had an impact on the induced immune response.

In live infections of *S. mansoni* eggs penetrate the small intestine to reach the intestinal lumen. Although the pathology of schistosomiasis is caused by lodged eggs within the intestine tissues (Wilson et al., 2007), immune cells can also sample luminal antigen. Dendritic cells can extend their dendrites into the intestinal lumen to directly sample antigen or soluble antigen can be transported across the epithelium into the lamina propria, where it is then sampled (Rescigno et al., 2001; Farache et al., 2013). To investigate whether luminal egg antigen could also

induce immune responses we injected eggs into the intestinal lumen and compared the induced responses to subserosal egg injections. We observed that, in contrast to the subserosal injection, the cluster of eggs was not visible after injection nor at the time of MLN harvest. 5 days after immunization MLNs were harvested and prepared for *in vitro* restimulation. After 3 days cytokines were measured by ELISA in the supernatants. We observed that both the subserosal and luminal injection of eggs induced antigen specific immune responses. The amount of detected IFN-γ was comparable. Concentrations of IL-4, IL-5 and IL-13, however, appeared somewhat, but not significantly, lower in luminal egg injection cell cultures (Fig. 3-3 A). These data suggested that luminal egg injections, despite being less relevant in a physiological context, can also induce antigen specific immune responses in the MLNs.

Non-viable *S. mansoni* eggs and the derived soluble egg antigen (SEA) have been reported to contain different compositions of antigen, but the immunologically relevant antigens, that have so far been identified are present in both preparations (Silva-Moraes et al., 2014; Everts et al., 2009). Both eggs and SEA have been used to induce Th2 responses in experimental settings (MacDonald et al., 2002; Klaver et al., 2015). To compare their effectiveness in our intestinal immunization model we injected either non-viable *S. mansoni* eggs or soluble egg antigen into the subserosa. 1,000 non-viable eggs in 20 µl PBS were compared to 20 µg SEA. 5 days after immunization MLN cells were harvested and 3-day restimulation cultures were set up. We observed that the injection of eggs and SEA induced very similar immune responses in the MLNs, with very similar amounts of IFN-γ, IL-4, IL-5 and IL-13 being detected (Fig. 3-3 B). We hypothesized that SEA injections would induce a slightly lower response, due to the soluble and more transient character of the antigen, but did not make this observation. Therefore, we concluded that the subserosal injection of SEA is, in our hands, as potent in inducing antigen specific immune responses in the MLNs as injected eggs.

We had observed that the subserosal injection of eggs induced immune responses in the MLNs. Therefore, some immunogenic component of egg antigen needed to be transported from the lamina propria to the draining lymph nodes, to be presented to naive T cells. To verify this, we compared immune responses induced by subserosally injected eggs to the responses induced by eggs that were directly injected into the MLN. Hence, we injected 1,000 eggs into the small

intestinal subserosa or into the subcapsular sinus of the MLN, as described previously (Cerovic et al., 2015). The cluster of eggs in both experiments was clearly visible after injection and 5 days after immunization. After 5 days, MLNs were harvested and restimulated with SEA for 3 days. We observed that the levels of the Th2 associated cytokines IL-4, IL-5 and IL-13 were comparable between the two types of injections. However, the amount of IFN-γ was 6-fold higher in MLN-injected mice compared to subserosally-injected animals (Fig. 3-3 C). The different response between subcapsular and subserosal injection suggested that locally available antigen was processed in a different way. Presumably, lamina propria DCs sampled subserosally injected antigen and migrated to the MLNs in order to present it whereas subcapsular egg antigen was likely taken up and presented by lymph node resident DCs.

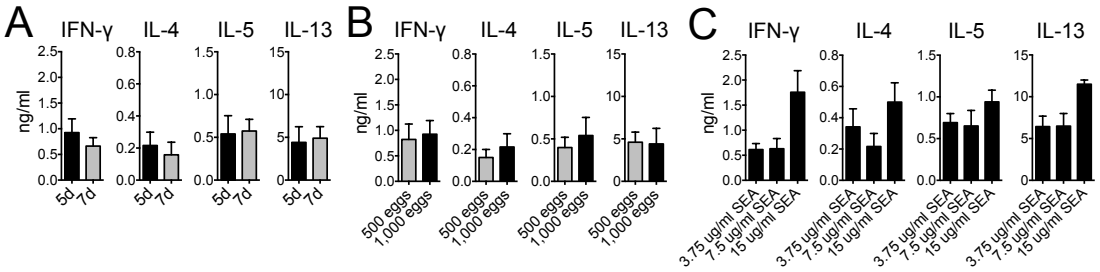

Figure 3-4. Optimization of experimental egg immunization protocol defines experimental parameters

To optimize our experiments, different parameters of the experimental egg immunization protocol were investigated. First, immune responses were compared 5 or 7 days after egg injection, and levels of cytokines were measured 3 days after restimulation (A). The restimulated cytokine response was also compared between mice that were injected with 500 or 1,000 non-viable eggs (B). Using MLNs harvested 5 days after injection, and restimulated for another 3 days, MLN cells were restimulated with different amounts of SEA and IFN-γ, IL-4, IL-5 and IL-13 were measured in the supernatants (C). Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests (A&B) or Kruskal-Wallis tests (C) were applied for statistical analysis.

Up to this point the experimental procedure involved the injection of 1,000 non-viable eggs, an immunization period of 5 days and restimulation of MLN cells for 3 days with 7.5 µg/ml SEA. These parameters had been adapted from previous publications (MacDonald et al., 2001; MacDonald and Pearce, 2002) and protocols available from our collaborator Andrew MacDonald. However, most of the available data were based on other tissue injection sites, especially the footpad,

and were not optimized for the intestine. The immunization period was one of the parameters that required validation. To focus on early immune induction events the optimal period of immunization would be the shortest time that robust immune responses would develop in the draining lymph nodes. Published data suggested a period between 5 and 7 days (MacDonald et al., 2001; MacDonald and Pearce, 2002). We therefore injected 1,000 non-viable eggs into the small intestinal subserosa of two groups of mice. One group was immunized for 5 days, which was the immunization period we had used in our previous experiments, the other group was immunized for 7 days. MLNs were harvested afterwards and restimulated with 7.5 µg/ml SEA for 3 days. The amount of cytokine that was produced in these restimulation cultures was almost identical between the two groups (Fig. 3-4 A), indicating that both 5 and 7 days were suitable periods to induce a robust T cell response against the injected eggs.

Another parameter that was not optimized was the number of injected eggs. Here, the lowest number of eggs that induced a robust immune response would be preferable, as this would decrease the chances of any unintended off-target effects. Furthermore, it would reduce the amount of material needed for each experiment. In the footpad the injection of 2,500 eggs had previously been used (MacDonald et al., 2001; MacDonald and Pearce, 2002). We had already reduced this number to 1,000 eggs for intestinal injections and tested if it could be reduced even further. We injected 500 or 1,000 non-viable eggs into the subserosal layer of the small intestine, harvested the MLNs after 5 days and restimulated them for an additional 3 days with 7.5 µg/ml SEA. We observed that the injection of 500 eggs was less consistent compared to 1,000 eggs and that the cluster of injected eggs was not visible after injection in all cases. However, the cytokines IFN-γ, IL-4, IL-5 and IL-13 could all be detected in restimulation cultures after the injection of 500 eggs, indicating that they induced a comparable immune response (Fig. 3-4 B). However, the amount of cytokine was slightly lower and more variable compared to the injection of 1,000 eggs, although the differences were not statistically significant. On balance, we concluded that the injection of 1,000 eggs was the optimal amount for intestinal injection inducing a robust and consistent immune response.

The last parameter we optimized was the amount of SEA used during restimulation. Again, we aimed for a concentration that would optimally restimulate

MLN cells *in vitro*, while minimizing the amount of material used. Previous publications had used 15 µg/ml of SEA during restimulation (MacDonald et al., 2001; MacDonald and Pearce, 2002). We had already reduced this amount to 7.5 µg/ml and tested if it could be reduced even further. We injected 1,000 cells into the subserosal layer of the small intestine, harvested the MLNs after 5 days and restimulated these cells with varying concentrations of SEA ranging from 15 µg/ml and 7.5 µg/ml to 3.75 µg/ml. We observed that all three concentrations could successfully restimulate T cells *in vitro* and that similar amounts of cytokines were produced (Fig. 3-4 C). It was surprising that the 4-fold lower concentration of SEA, compared to the literature, was almost as potent in restimulating T cells as higher concentrations. We could however observe that the amount of cytokines produced with 3.75 µg/ml SEA was lower compared to 15 µg/ml, suggesting a linear relationship between the concentration of SEA used and the amount of cytokines detected. However, this difference was not statistically significant. From this experiment we concluded that 7.5 µg/ml SEA was the optimal concentration for restimulating MLN cells, which produced an equally consistent response as published concentrations but reduced the amount of SEA needed for each experiment. We had therefore tested important experimental parameters and optimized our intestinal egg immunization protocol.

3.1.2 T cells responses against egg antigens are primed in the MLNs

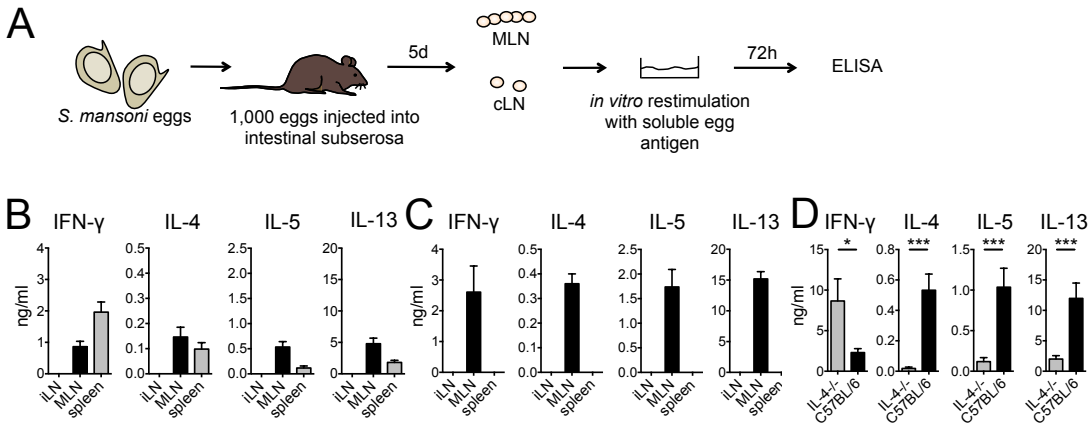

Figure 3-5. Intestinal egg injection induces classical Th2 immune responses in the draining MLNs and not in other lymphoid organs

Based on our previous findings a standard experimental egg immunization protocol was defined. 1,000 non-viable *S. mansoni* eggs were subserosally injected into the intestine. After 5 days of immunization lymph nodes were harvested and restimulated with 7.5 µg/ml SEA for 72 hours. Released cytokines were then measured in the supernatants by ELISA. The experimental scheme is shown in (A). Immune responses were measured in restimulation cultures from inguinal lymph nodes (iLNs), MLNs and the spleen (B). Similarly, cytokines were measured in the same tissues 3 days after restimulation. Here, mice were treated with 1.5 µg FTY720 during day 1 and day 3 of the immunization (C). Amounts of IFN-γ, IL-4, IL-5 and IL-13 were measured in MLN restimulation cultures of egg injected homozygous KN2 (IL-4-/-) or C57BL/6 mice (D). Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests (D) were applied for statistical analysis (*P ≤ 0.05; ***P ≤ 0.001).

Based on these results we defined a standard experimental protocol for intestinal injections, whereby the subserosal injection of 1,000 eggs with an immunization period of five days followed by a 3-day restimulation period with 7.5 µg/ml SEA would result in a robust Th2 response in the draining lymph nodes (Fig. 3-5 A). Using this standard egg injection protocol we injected 1,000 non-viable eggs subserosally into the small intestine and harvested inguinal lymph nodes (iLN), mesenteric lymph nodes (MLN) and the spleen after 5 days. Cell suspensions were restimulated with 7.5 µg/ml SEA for 3 days and immune responses characterized by measuring the cytokines released in the supernatants of these cultures. We hypothesized that immune responses should only occur in the MLNs, and not the iLNs or the spleen, as the MLNs were the only tissue directly draining the injection site. As expected we could detect IFN-γ, IL-4, IL-5 and IL-13 in the supernatants of MLN restimulation cultures at levels comparable to those measured in previous experiments. As expected, we could not detect any

cytokines in restimulation cultures of iLN cells, but observed robust cytokine responses in spleen restimulation cultures. Here, the level of IFN-γ responses were higher and Th2 cytokines lower compared to the MLNs, but Th2 cytokines were clearly detectable suggesting the presence of primed Th2 cells in the spleen (Fig. 3-5 B).

To address whether antigen was directly transported to the spleen or if recirculating primed T cells were responding to *in vitro* restimulation we combined intestinal egg injections with the administration of FTY720. This molecule inhibits the egress and recirculation of T cells from lymph nodes (Brinkmann et al., 2004), and would therefore clarify if splenic immune responses were due to the recirculation of T cells or direct priming in the spleen itself. During the 5 day immunization period 1.5 µg FTY720 in 30 µl DMSO was injected i.p. on days 1 and 3. MLNs, iLNs and spleens were harvested on day 5 and restimulated with SEA. We could not detect any cytokines produced by iLN or spleen cells in the supernatants of these cultures. Furthermore, we observed that the level of IFN-γ, IL-4, IL-5 and IL-13 responses from MLN cells had all increased up to 3-fold, compared to non-treated egg injections (Fig. 3-5 C). These results clearly indicated that the responses observed in the spleen were due to recirculating T cells that had been primed in the MLNs and not directly primed in the spleen. By blocking the egress of these primed cells by administrating FTY720, primed T cells accumulated in the MLNs, which increased the observed immune responses.

These immune responses are controlled by key cytokines. IFN-γ is involved in many proinflammatory conditions, whereas IL-4 is a key cytokine of the type 2 immune response and is produced by Th2 cells. It is an important signalling molecule and affects other effector cells such as B cells, but more importantly is required for the differentiation of naive T cells into Th2 cells. It also has an impact on other T helper cells and limits the development of Th1 cells and the production of IFN-γ (Constant and Bottomly, 1997). Thus, in the absence of IL-4 or its receptor IL-4R, Th2 responses are inhibited (Jankovic et al., 2000). To verify that the immune responses we observed upon egg injections into the intestine were indeed driving an IL-4 dependent Th2 response we used homozygous KN2 mice ($IL\text{-}4^{-/-}$), which did not express functional IL-4 (Mohrs et al., 2005). 5 days after injection MLNs were harvested and immune responses were quantified by measuring the cytokines IFN-γ, IL-4, IL-5 and IL-13 in supernatants 3 days after

restimulation with SEA. We could only detect minute levels of the Th2 associated cytokines IL-4, IL-5 and IL-13 in the supernatants of IL-4$^{-/-}$ mice, which were at least 10-fold lower than the responses observed in C57BL/6 mice. Furthermore, IFN-γ was present at increased amounts, with levels 4-fold higher than observed in C57BL/6 MLN cell cultures (Fig. 3-5 D). These observations indicated that the injection of *S. mansoni* eggs in the intestine induced a genuine Th2 response in the draining lymph nodes, which produced IL-4, IL-5 and IL-13 and could not develop in IL-4 deficient mice. As Th2 cells also actively control and inhibit Th1 responses (Pearce et al., 1991), the absence of these cells in IL-4$^{-/-}$ mice, led to an increase in IFN-γ levels.

3.1.3 CD4 T cells secrete cytokines detected in restimulation cultures

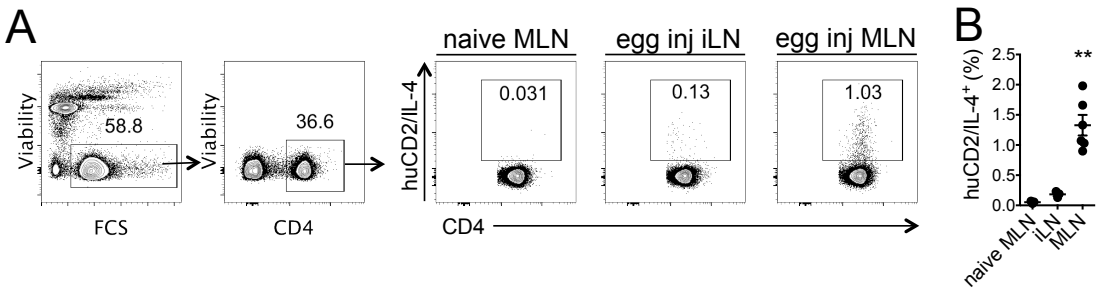

Figure 3-6. Heterozygous KN2 mice report IL-4 secretion of CD4 T cells in the MLNs of egg injected mice

Cells were harvested from heterozygous KN2 mice and stained for flow cytometry. Live CD4$^+$ cells were selected and the expression of huCD2, which reports IL-4 secretion in KN2 mice, measured in MLN cells from naive mice and iLN and MLN cells from egg injected mice (A). Six mice were analysed in total and the percentage of huCD2/IL-4$^+$ CD4 T cells presented in B. Data represent two independent experiments (mean ± SEM) with each point representing one animal. Kruskal-Wallis tests (B) were applied for statistical analysis (**P ≤ 0.01).

To confirm that the type 2 cytokines we measured in MLN restimulation cultures were indeed secreted by CD4$^+$ Th2 cells we again used the KN2 mouse strain, which can also be used to detect cell-specific IL-4 secretion using flow cytometry. In this mouse strain the first two exons of IL-4 are replaced by human CD2 (huCD2) (Mohrs et al., 2005). Upon expression and secretion of IL-4 the reporter gene huCD2 is produced instead and expressed as a cell surface marker, which can be detected with a monoclonal antibody. Therefore, cells that were actively

secreting IL-4 label positive for huCD2, which allows a precise understanding of active Th2 responses. In heterozygous KN2 mice one allele serves as the huCD2 reporter allele, while the wild type allele ensures normal IL-4 production. Heterozygous mice have been reported to not have any IL-4-related defects, whereas homozygous mice do not produce any functional IL-4 and are effectively IL-4 deficient (Mohrs et al., 2005).

To study cellular IL-4 secretion, we injected heterozygous KN2 mice with *S. mansoni* eggs following our standard egg injection protocol and harvested inguinal lymph nodes and MLNs from egg injected and naive mice five days after immunization. Instead of restimulating these cells with SEA we stained the cell suspensions with monoclonal fluorescently labelled anti-CD4 and anti-huCD2 antibodies and used a viability dye to exclude dead cells from analysis. By gating on live $CD4^+$ cells, we selected all CD4 T cells and assessed their secretion of IL-4 by detecting the expression of huCD2 (Fig. 3-6 A). Flow cytometric analysis revealed that no huCD2 expressing cells could be detected in naive MLNs or inguinal LNs of egg injected mice. However, in the MLNs of egg injected animals $CD4^+$ cells were the only cells that expressed huCD2. 1.5% of all $CD4^+$ T cell expressed huCD2, indicating that a substantial number of CD4 T cells secreted IL-4 and had differentiated into Th2 cells after egg injection (Fig. 3-6 B). These observations were in line with our previous findings using restimulation cultures and confirmed that T cells are only primed upon egg injection in the draining lymph nodes, where they differentiate into IFN-γ-producing cells and IL-4-secreting Th2 cells.

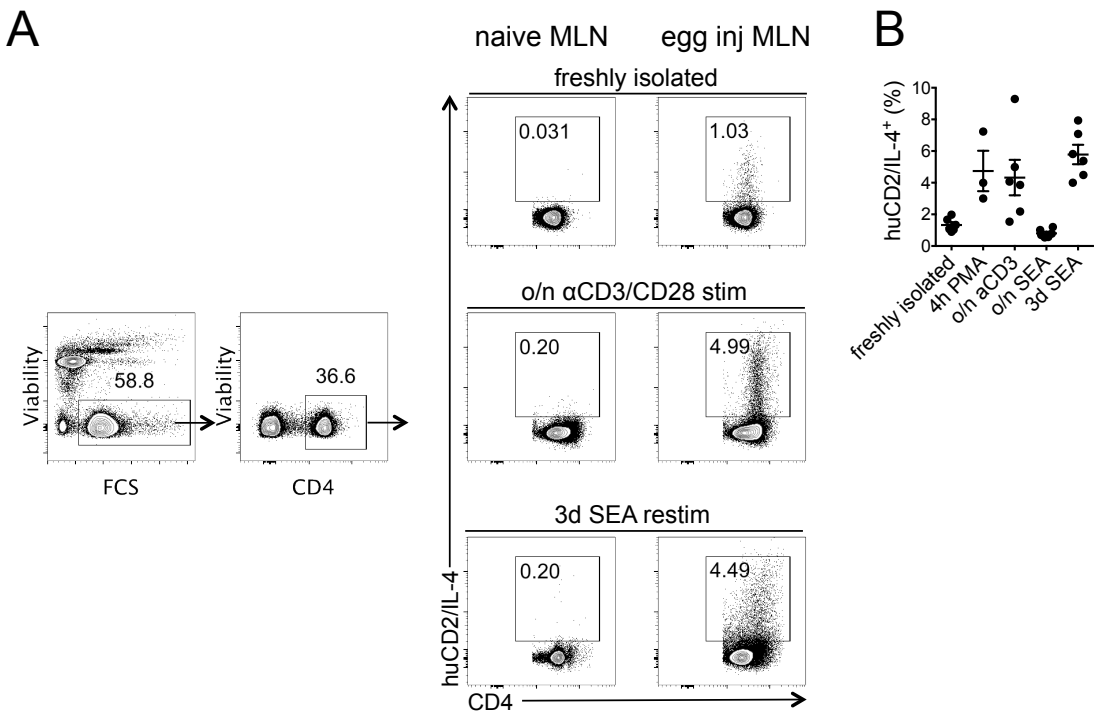

Figure 3-7. Different cell stimulation protocols promote differences in IL-4 secretion of CD4 T cells of egg injected heterozygous KN2 mice.

MLN cells were harvested from heterozygous KN2 mice 5 days after egg injection. Cells were subjected to different cell stimulation protocols or stained directly for flow cytometry. Cell stimulations included 2.5 ng/ml PMA and 1 µg/ml ionomycin for 4 hours (4h PMA), 5 µg/ml plate-bound anti-CD3 and 2 µg/ml soluble anti-CD28 overnight (o/n aCD3) or 18 hours (o/n SEA) or 72 hours (3d SEA) of restimulation with 7.5 µg/ml SEA. $CD4^+$ T cells were selected from all live cells and their secretion of IL-4 measured by the detection of the reporting surface marker huCD2 (A). Two independent experiments were performed and data was pooled and presented as the percentage of $huCD2^+$ CD4 T cells in panel B. Data represent two independent experiments (mean ± SEM) with each point representing a data point from an individual animal.

Having established that heterozygous KN2 mice could be used to monitor active IL-4 secretion by Th2 cells we used this method to compare different *in vitro* cell stimulation protocols to assess whether IL-4 secretion would differ between antigen specific and non-specific restimulation. We injected 1,000 non-viable eggs into the subserosal layer of the small intestine of heterozygous KN2 mice, harvested the MLNs after five days and subjected them to different *in vitro* cell stimulation and restimulation protocols. One group was treated with 2.5 ng/ml PMA and 1 µg/ml ionomycin for 4 hours, a common method used for intracellular flow cytometry staining. Another group was stimulated with 5 µg/ml plate-bound anti-CD3 and 2 µg/ml soluble anti-CD28 overnight. PMA/ionomycin and anti-CD3/anti-CD28 stimulation are both antigen non-specific ways to stimulate T cells and have extensively been used in the literature. These stimulation methods were

compared to freshly isolated cells, overnight restimulation with 7.5 µg/ml SEA and 3 day restimulation with 7.5 µg/ml SEA. After each treatment cells were stained with fluorescently labelled anti-CD4 and anti-huCD2 antibodies and a viability dye was used to exclude dead cells from analysis. Cells survival was comparable between the different *in vitro* restimulation protocols and live $CD4^+$ T cells were assessed for their secretion of IL-4 by measuring the expression of huCD2 (Fig. 3-7 A). All *in vitro* stimulation protocols, excluding overnight restimulation with SEA, induced increased frequencies of IL-4 secreting Th2 cells, compared to the freshly isolated control group (Fig. 3-7 B). Comparable amounts of IL-4 secreting T cells between antigen specific restimulation with SEA and non-specific stimulation with PMA/ionomycin or anti-CD3/anti-CD28 showed that both methods were sufficient to stimulate and detect Th2 cells primed after egg injection.

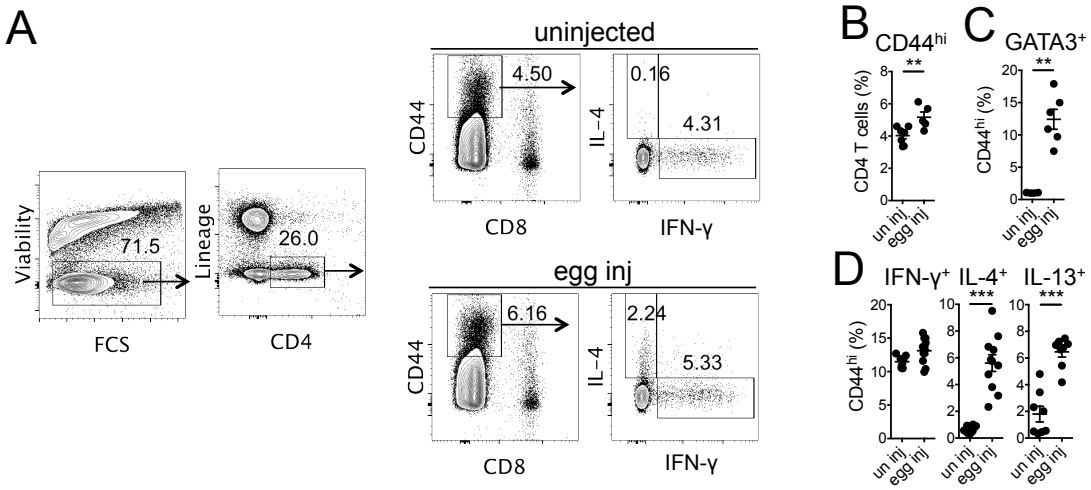

Figure 3-8. Stimulated $CD44^{hi}$ CD4 T cells from the MLN express GATA3 and produce IL-4 and IL-13 after egg injection

MLN cells from uninjected (un inj) and egg injected (egg inj) C57BL/6 mice were harvested after 5 days of immunization and stimulated with 2.5 ng/ml PMA, 1 µg/ml ionomycin and 0.5% GolgiStop for 4 hours. Cells were then stained for cell surface markers, fixed and permeabilized and stained intracellularly. Live cells were selected and $CD44^{hi}$ CD4 T cells were analysed for their expression of GATA3 or the production of cytokines (A). Percentages of $CD44^{hi}$ CD4 T cells were compared between naive and egg injected animals (B). The expression of GATA3 (C) and the production of IFN-γ, IL-4, IL-5 (D) by $CD44^{hi}$ CD4 T cells from naive and egg injected mice was measured. Data represent at least two independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied for statistical analysis (**P ≤ 0.01; ***P ≤ 0.001).

We assumed that the IFN-γ we had detected in *in vitro* restimulation cultures was produced by Th1 cells, whereas IL-4, IL-5 and IL-13 were produced by Th2 cells.

However, we had not identified the cellular source of these cytokines, except for IL-4, which was exclusively secreted by CD4 T cells after egg injection. Having established that cell stimulation with PMA/ionomycin was sufficient to optimally stimulate heterozygous KN2 cells, we hypothesized that this stimulation would also be sufficient to detect other cytokines by flow cytometry.

We injected eggs into the serosa of the small intestine, harvested the MLNs after 5 days and stimulated MLN cell suspensions from naive and egg injected mice with 2.5 ng/ml PMA and 1 µg/ml ionomycin for 4 hours. In addition, we added 0.5% GolgiStop solution, which prevents cytokine secretion and allows the intracellular detection of the produced cytokines. After stimulation we stained the cells with Fixable Viability Dye eFluor780 and fluorescently labelled antibodies against the cell surface markers CD4, CD8 and CD44. Cells were fixed and permeabilized using the eBioscience Foxp3/Transcription Factor Staining Buffer Set and stained for the intracellular cytokines IFN-γ, IL-4 and IL-13 and the Th2 associated transcription factor GATA3 (Amsen et al., 2004). Cells were gated on live $CD44^{hi}$ CD4 T cells, that represented activated T cells, and we assessed the percentage of cytokine expressing cells (Fig. 3-8 A). We observed a significant increase of $CD44^{hi}$ cells in egg-injected mice compared to naive controls (Fig. 3-8 B), which demonstrated that naive T cells had been primed and activated after egg injection. In naive animals the percentage of GATA3 expressing cells was negligible, whereas upon egg injection around 12% of activated T cells expressed GATA3 (Fig. 3-8 C). This indicated that no activated Th2 cells were present in naive animals but were specifically induced upon egg injection, which was consistent with our results from *in vitro* restimulation cultures. We also observed a strong increase of IL-4 and IL-13 producing T cells after egg injection along with a slight increase of IFN-γ^+ cells (Fig. 3-8 D). IFN-γ^+ cells did not co-express IL-4 or IL-13, whereas IL-4 and IL-13 were produced by overlapping cell populations. This indicated that in response to egg antigens IFN-γ-producing cells as well as Th2 cells were induced.

3.1.4 Injections of *S. mansoni* eggs into different segments of the murine intestine induce immune responses in specific MLNs

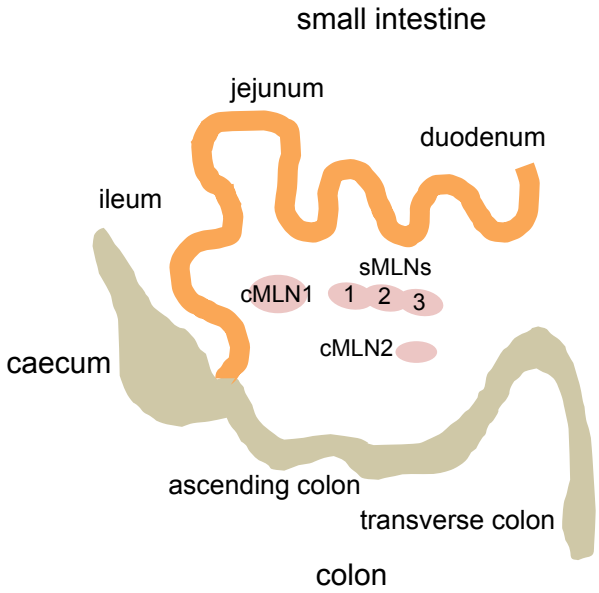

Figure 3-9. Anatomical segments of the murine gastrointestinal tract

The small intestine, caecum and colon are drained by individual MLNs. These were identified as sMLN1-3 and cMLN1/2. Anatomical segments of the small intestine included the duodenum, jejunum and ileum, whereas the colon was divided into the ascending, transverse and descending colon (not shown).

Having confirmed that small intestinal *S. mansoni* egg injections are a suitable model to induce Th2 responses in the draining lymph nodes, we tested whether the injection of *S. mansoni* eggs would also result in Th2 responses in other segments of the gastrointestinal tract.

The small intestine and colon have evolved as separate organs, with a distinct anatomy, that serves physiologically distinct functions such as nutrient uptake and water resorption, harbour distinct populations of microbiota, have evolved distinct immune systems and can be affected by distinct pathologies including parasites that either populate the small intestine or colon

(Mowat and Agace, 2014; Grencis, 2015). Both organs drain into anatomically separate MLNs: the small intestinal sMLNs and colon draining cMLNs (Houston et al., 2016). The sMLNs can further be divided into at least three easily distinguishable lymph nodes that are arranged in a chain (Fig. 3-9). We have here termed these separate lymph nodes sMLN1, 2 and 3. We had not previously tested whether these individual sMLNs also drain anatomically distinct segments of the small intestine, which can further be subdivided into the duodenum, jejunum

and ileum. The small intestine is followed by the caecum and the ascending, transverse and descending colon, which are drained by the colon draining lymph nodes cMLN1 and cMLN2 and other lymph nodes distributed within the body cavity (Carter and Collins, 1974).

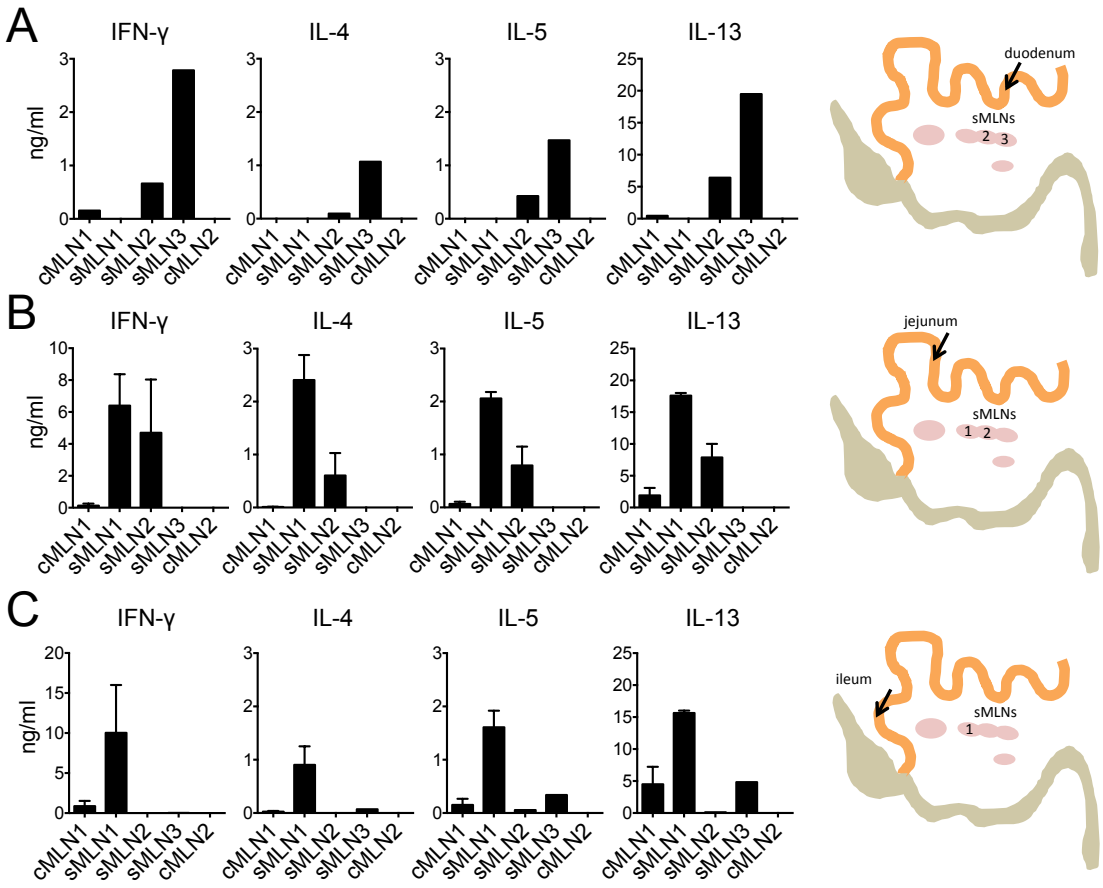

Figure 3-10. Distinct anatomical segments of the small intestine are drained by individual sMLNs

The duodenum (A), jejunum (B) and ileum (C) were injected with 1,000 *S. mansoni* eggs. The approximate injection site is marked with an arrow. FTY720 was administered at days 1 and 3. Individual MLNs were harvested 5 days after immunization and restimulated for 3 days with SEA. Cytokine responses were measured in each individual lymph node and responding lymph nodes highlighted in each corresponding schematic. Data represent at least two independent experiments (mean ± SEM) with at least three animals per group.

In order to understand whether different segments of the gastrointestinal tract could mount similar immune responses against injected *S. mansoni* eggs, eggs were injected in different parts of the small intestine and colon. We predicted that these responses would develop in distinct MLNs that selectively drained specific anatomical segments of the intestine. Therefore, all mesenteric lymph nodes were

harvested individually 5 days after egg injection and restimulated with SEA for another 3 days. During immunization FTY720 was administered at day 1 and day 3 to prevent T cell egress and recirculation. This guaranteed that the detected cytokines were generated by T cells primed in the harvested draining lymph node, and not by recirculating T cells that had originally been primed at another site. By analysing the produced cytokines in restimulation cultures of all the individual MLNs we were able to identify which draining lymph nodes responded and thus drained the injected segment of the intestine. In general we observed that egg injections into all segments of the intestine generated antigen-specific immune responses against injected eggs, and that these immune responses developed in distinct draining lymph nodes. In particular, egg injections in the duodenum resulted in strong responses in sMLN3 and to a smaller extent in sMLN2 (Fig. 3-10 A). The jejunum was drained by sMLN1 and sMLN2, where we detected the cytokines IFN-γ, IL-4, IL-5 and IL-13 (Fig. 3-10 B) and injections in the ileum resulted in T cells responses predominantly in the lymph node sMLN1 (Fig. 3-10 C). All injections in different segments of the small intestine induced comparable levels of Th2 responses measured by the amount of cytokines released during restimulation and we concluded that the anatomical segments of the small intestine were drained by individual small intestinal draining lymph nodes. In particular, the duodenum was drained by sMLN3, the jejunum drained into sMLN1/2 and the ileum was drained by sMLN1.

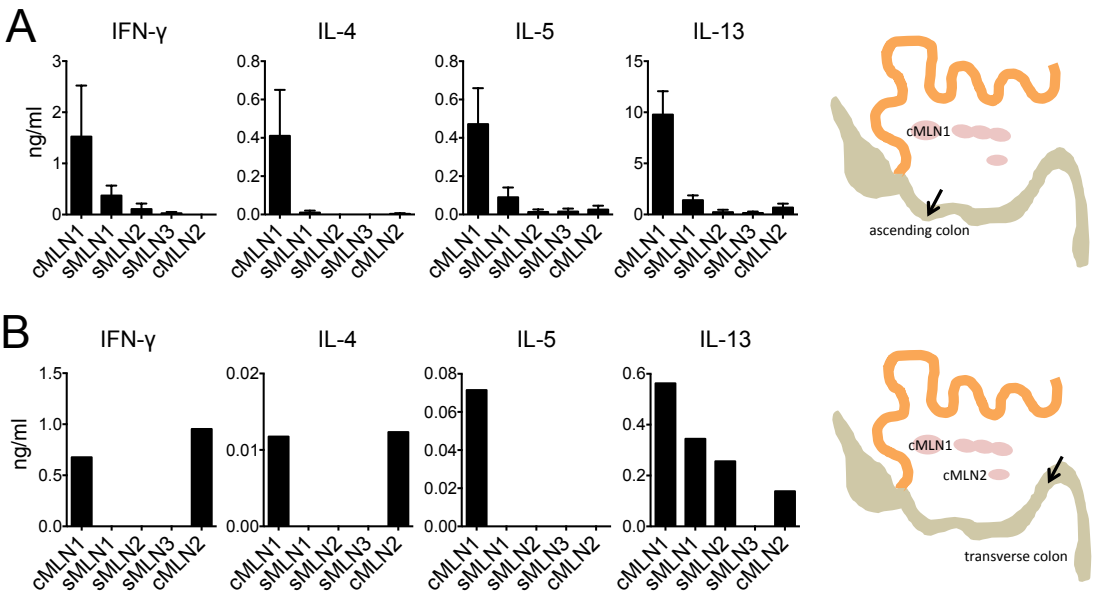

Figure 3-11. Egg injection into the colon induced immune responses in the cMLNs

1,000 non-viable *S. mansoni* eggs were injected into the ascending (A) and transverse (B) colon. The approximate injection site is marked with an arrow. FTY720 was administered at days 1 and 3. Individual MLNs were harvested 5 days after immunization and restimulated for 3 days with SEA. Cytokine responses were measured in each individual lymph node and responding lymph nodes highlighted in each corresponding schematic. Data represent at least two independent experiments (mean ± SEM) with at least three animals per group.

Similarly, eggs were injected into different parts of the colon. The descending colon was excluded from further analysis, as it was not accessible during the surgical procedure used for egg injection. 1,000 non-viable eggs were injected into the ascending or transverse colon and FTY720 was administered at day 1 and day 3. After 5 days individual MLNs were harvested and restimulated with SEA for 3 days and immune responses were detected by measuring the released cytokines in the supernatants. Egg injections in the ascending colon resulted in immune responses in cMLN1 (Fig. 3-11 A). The levels of IFN-γ, IL-4, IL-5 and IL-13 were slightly lower compared to values measured upon small intestinal injection. However, they indicated that *S. mansoni* eggs could not only induce robust T cell responses in the small intestine but also in the colon. Cytokines were also detected in cMLN1 and cMLN2 upon transverse colon egg injection, however to a much lower extend (Fig. 3-11 B). We hypothesize that these low responses were due to inappropriate egg injections in the transverse colon and did not represent tissue-specific deficiencies in T cell priming.

We thus concluded that *S. mansoni* egg injections also represented a suitable model to induce parasite-specific Th2 immune responses in the colon, which was drained by specific cMLNs.

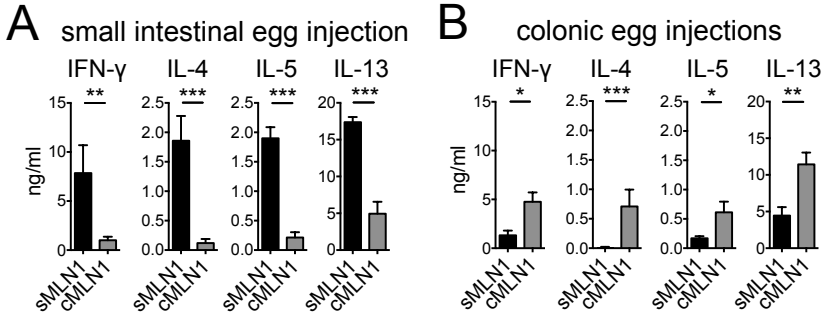

Figure 3-12. Egg injections into the ileum induce immune responses in sMLN1 whereas immune responses against egg injections into the ascending colon develop in cMLN1

1,000 non-viable eggs were injected into the ileum (A) or the ascending colon (B). 5 days after immunization the lymph nodes sMLN1 and cMLN1 were harvested and restimulated with SEA for 3 days. IFN-γ, IL-4, IL-5 and IL-13 were measured in the supernatants of these restimulation cultures. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

Having established that distinct anatomical segments of the intestine were drained by specific individual lymph nodes we repeated this experiment without the administration of FTY720. We had previously observed that five days after immunization recirculating T cells had not yet migrated to other lymph nodes, like the iLNs, suggesting that recirculation to other lymph nodes is unlikely within that time. To compare small intestinal and colonic immune responses we chose representative regions of each tissue that were readily accessible during surgery and were physiologically relevant. The ileum of the small intestine is heavily affected by penetrating *S. mansoni* eggs during live infection (Pearce and MacDonald, 2002), and was thus chosen to represent the small intestine in this and further experiments. The ascending colon was the only region of the colon that was readily accessible during surgery and mounted robust immune responses against injected eggs. Hence, it was chosen as the injection site for the colon. 5 days after egg injection in the ileum or the ascending colon individual MLNs were harvested. Previous experiments indicated that the ileum was predominantly drained by sMLN1 and the ascending colon by cMLN1. Therefore, only these two lymph nodes were collected. After a 3-day restimulation with SEA, supernatants

were assessed for secreted cytokines by ELISA. We observed that the responses were comparable to our previous findings, where we had administered FTY720. This was consistent with our hypothesis that recirculation of T cells into other lymph nodes does not occur within the 5 days of immunization. After egg injection into the ileum high levels of IFN-γ, IL-4, IL-5 and IL-13 were detected in sMLN1, with only minor concentrations detectable in cMLN1 (Fig. 3-12 A). Conversely, egg injections in the ascending colon predominantly induced immune responses in cMLN1 and not in sMLN1 (Fig. 3-12 B). Thus, our observations clearly indicated that parasite antigen could induce strong Th2 responses both in the small intestine and the colon, which are each drained by specific MLNs, and that *S. mansoni* egg injections are a suitable model to study the cellular mechanisms driving Th2 responses in both organs.

3.2 Discussion

We have established a novel and physiologically relevant method of *S. mansoni* egg immunization in the intestine. This method of injecting non-viable eggs into the subserosal layer of the small intestine and colon will allow us to specifically investigate the immune responses mounted against parasite antigens in the intestine and study their induction. The induction of Th2 responses from the intestine has been largely overlooked despite the facts that *S. mansoni* eggs predominately affect the small intestine during live infections, and that most parasites invade the gastrointestinal tract (Grencis et al., 2014). We have shown that eggs induced strong antigen specific immune responses in the draining lymph nodes, where *in vitro* restimulated MLN cells secreted IFN-γ, IL-4, IL-5 and IL-13, which are commonly induced by *S. mansoni* eggs (MacDonald and Pearce, 2002; MacDonald and Maizels, 2008; Cook et al., 2015) (Fig. 3-2).

In live infections of *S. mansoni,* eggs penetrate the small intestinal wall to reach the intestinal lumen. Some of these eggs become lodged within the intestine, where they induce strong immune responses, lead to granuloma formation and drive the pathology of schistosomiasis (Wilson et al., 2007). Most eggs however reach the intestinal lumen and are excreted and we observed that injections of eggs into the small intestinal lumen could also induce similar immune responses compared to injections into the intestinal subserosa. This suggested that antigen from luminally-injected eggs were detected by the immune system and induced antigen specific immune responses in the MLNs. Reported mechanisms by which lamina propria immune cells sample luminal antigen include dendritic cells that extend their dendrites into the intestinal lumen or the transport of soluble antigen across the epithelium into the lamina propria (Farache et al., 2013). However, uptake of luminal antigen is not an efficient process and mostly involves professional phagocytes such as macrophages or M cells located in the Peyer's patches. Furthermore, limited antigen availability and its more transient presence, due to the continuous movement of luminal content, reduce antigen uptake from the lumen (Mowat and Agace, 2014). Surprisingly, we observed few differences of the induced immune responses from luminal egg injections compared to subserosal injections (Fig. 3-3 A). However, it needs to taken into account that during the injection into the lumen the intestinal tissue was penetrated and the epithelium damaged, which might have facilitated the entry of egg antigen into the

lamina propria. Thus, our luminal injection of eggs more closely resemble eggs that penetrate the intestine and reach the lumen, creating tissue damage along the way, and is less representative of the uptake of luminal antigen by intact intestinal tissue. We therefore chose to continue our experiments using the subserosal injection of antigen as we considered it likely that this would generate more robust and consistent immune responses. Subserosal injections also better represent trapped eggs within the intestinal subserosal tissue, where they elicit type 2 immune responses during live infection, lead to granuloma formation and cause fibrotic lesions, which are all key features of chronic schistosomiasis (Wynn et al., 2004).

Interestingly, we observed that the injection of eggs in other tissues, such as the MLN, caused higher proinflammatory responses, measured by the amount of IFN-γ produced after restimulation, whereas Th2 responses were comparable (Fig. 3-3 C). Our interpretation of these results was that after subserosal injection lamina propria DCs sampled egg antigens and migrated to the MLNs in order to present them to and prime naive T cells. In the experiments that follow, we have indeed shown that migrating DCs take up subserosally injected egg antigen and transport it to the draining lymph nodes, where they induce immune responses. Contrarily, egg antigen that was injected under the MLN subcapsule was likely taken up and presented by lymph node resident DCs. Our observation that type 1 immune responses were significantly increased when eggs were directly injected into the MLN suggests that an inhibiting factor, that specifically inhibits proinflammatory responses, was not present in the MLNs. Indeed a tolerogenic environment exists within the intestinal lamina propria, which dampens intestinal proinflammatory immune responses, through the presence of regulatory T cells, the production of IL-10 and tolerogenic macrophages (Mowat and Agace, 2014). This allows the intestinal immune system to tolerate harmless proinflammatory stimuli from the microbiota and food antigens, and can also affect immune responses to inert antigens, e.g. during oral tolerance. We suggest that proinflammatory responses against egg antigens were also suppressed within the intestinal microenvironment. However, as we did not observe a downregulated response of type 2 immune responses, we suggest that they are not inhibited by this tolerogenic environment. This could ensure that intestinal parasite infections prompt an immediate reaction and induce potent Th2 responses.

We also observed that the injection of eggs into the intestine induced antigen specific immune responses in the draining lymph nodes and the spleen but not the inguinal draining lymph nodes (iLNs) (Fig. 3-5 B). This was surprising as the spleen does not directly drain the intestines but rather responds to systemic infections. During live infection of *S. mansoni* Th2 immune responses can be detected in locally affected organs such as the liver as well as systemically in the spleen (Dunne and Pearce, 1999). However, it has been reported in the house dust mite model of Th2 induction in the lung that bona fide immune responses both develop in lung draining lymph nodes and in the spleen. It was identified that this is due to the destruction of the lung epithelial barrier during infection, antigen transport in blood and priming of naive T cells against these antigens in the spleen. Thus, the administration of FTY720, which inhibits the egress and recirculation of T cells from lymph nodes (Brinkmann et al., 2004), did not affect splenic immune responses in this model (Plantinga et al., 2013).
In contrast, in our intestinal egg injection model splenic responses were no longer detectable after FTY720 administration, indicating that they were due to recirculating T cells (Fig. 3-5 C). A possible explanation why we did not observe recirculating T cells responses in the iLNs might be the low number of primed recirculating Th2 cells in the iLNs, which could have been under the detection limit of *in vitro* restimulation iLN cultures. Using heterozygous KN2 mice, which report IL-4 secretion, we indeed showed that only around 0.1% of CD4 T cells secreted IL-4 after subserosal egg injection in the iLNs, whereas around 1.3% secreted IL-4 in the MLNs (Fig. 3-6).

When using different *in vitro* T cell stimulation protocols to stimulate MLN cells from egg injected mice we observed that more huCD2 expressing cells were detected after PMA/ionomycin and anti-CD3/anti-CD28 stimulation, compared to the freshly isolated control group (Fig. 3-7 B). This increase indicated that these stimulation protocols induced active secretion of IL-4 in more cells compared to the non-treated controls, suggesting that not all Th2 cells that were primed actively secreted IL-4 after being freshly isolated. Furthermore, we observed that overnight restimulation with SEA was not sufficient to induce IL-4 secretion, as only a low percentage of cells responded. However, three days of restimulation induced a high percentage of IL-4 secreting $CD4^+$ T cells, which was comparable to the percentages observed with PMA/ionomycin and anti-CD3/CD28 stimulation. As SEA is a complex antigen that first needs to be processed by antigen presenting

cells and does not directly have an impact on T cells (Cervi et al., 2004), it was of no surprise that the overnight restimulation with SEA was ineffective. Thus, antigen specific restimulation by SEA and non-specific stimulation with PMA/ionomycin or anti-CD3/anti-CD28 were suitable to stimulate Th2 cells primed after egg injection *in vitro*.

Similarly we observed that 12% of activated $CD44^{hi}$ CD4 T cells expressed GATA3 after egg injection and PMA/ionomycin stimulation but only half of these cells (6% of activated $CD44^{hi}$ CD4 T cells) actively produced IL-4 and IL-13 (Fig. 3-8). This suggested that not all Th2-committed cells actively secreted IL-4 and IL-13, which we had observed in our IL-4 reporter mice. IFN-γ was not co-expressed with any of these Th2-associated markers suggesting that they were produced by separate Th1 cells. In contrast to the Th2 markers, IFN-γ secretion could already be detected in naive animals and only increased slightly after egg injection. Most likely PMA/ionomycin stimulation was sufficient to induce IFN-γ secretion in activated T cells that were already primed against other antigens, and the slight increase of IFN-γ after egg injection represented Th1 cells that were specifically primed against egg antigen. This would correspond to our findings that in MLN restimulation cultures IFN-γ, IL-4, IL-5 and IL-13 were all secreted in an antigen dependent manner.

We observed that specific anatomical segments of the gastrointestinal tract were drained by individual lymph nodes. Compared to previous experiments, where the entire MLN chain was harvested after egg injection, the concentration of IFN-γ, IL-4, IL-5 and IL-13 were increased in restimulation cultures of individually harvested lymph nodes (Fig. 3-10). This suggested that harvesting the entire MLN chain led to a dilution of primed T cells that were only present in an individual lymph node, reducing the restimulation response. However, we observed that injections into the colon resulted in more variable responses. Due to the arrangement of the intestines during egg injection surgery the descending colon could not be adequately reached. Furthermore, immune responses after egg injection in the transverse colon were much lower compared to those measured after injection into the ascending colon (Fig. 3-11). This relatively inadequate induction was most likely due to difficulties in accessing the transverse colon during egg injection. During the surgical procedure we observed that the correct placement of the needle into the subserosal tissue of the transverse colon was obstructed by surrounding tissue and that the cluster of eggs was not visible after injection.

Furthermore, Carter *et al.* demonstrated that the transverse colon is also drained by the pancreatic nodes, which were not harvested in these experiments (Carter and Collins, 1974).

We furthermore observed that the immune responses after colonic egg injections generated lower cytokine concentrations than those in the small intestine, suggesting that organ specific differences might exist (Fig. 3-12). A possible explanation is the different anatomical architecture of the colon. The thicker muscle layer might hinder immune cells to reach and take up injected egg antigens. It has also been reported that fewer DCs are present in the colonic lamina propria and the draining lymph nodes (Denning et al., 2011; Houston et al., 2016), which would reduce the number of migratory antigen presenting cells and might affect the immune response they induce in the colonic draining lymph nodes.

In general, we have demonstrated that different anatomical segments of the small intestine and colon were drained by individual lymph nodes, which were part of the MLN chain. This confirmed early findings by Carter *et al.* that an anatomical drainage network connects particular segments of the gastrointestinal tract to individual lymph nodes (Carter and Collins, 1974). To our knowledge we are the first to show that this network is important to drive appropriate immune responses and enables the intestine to mount fast and effective immune responses to local stimuli. Several G protein coupled receptors encoding CC-Chemokine ligands and receptors have been identified to influence T cell homing to the small intestine or colon (Habtezion et al., 2016). However, it remains to be determined whether T cell homing also occurs to individual segments within the small intestine and colon or if generally recruited effector T cells locally expand at the affected area.

3.3 Conclusions

We have established that *S. mansoni* egg injections into the intestine induce strong immune responses in the draining lymph nodes. Subserosal injection of eggs into the intestine thus serve as a model system that represents eggs that penetrated the intestine and became trapped within the lamina propria, leading to granuloma formation during live infection. Upon egg injection, IFN-γ, IL-4, IL-5 and IL-13 were produced in the draining lymph nodes. IL-4, IL-5 and IL-13 were hereby released by GATA3-expressing CD4 T cells that have differentiated into Th2 cells, recognizing egg antigens. Hereby, T cell priming occurred directly in the mesenteric lymph nodes and not in other lymphoid tissues, indicating the likely involvement of migratory antigen presenting cells that transport and present egg antigens in the MLNs.

Immune responses also developed after egg injection in the colon, specifically in the colon draining cMLNs. Further analysis revealed that different anatomical segments of the small intestine and colon were drained by individual lymph nodes, which were part of the MLN chain. These findings confirm the presence of an anatomical drainage network that connects segments of the gastrointestinal tract to individual lymph nodes. Thus, intestinal *S. mansoni* egg injections provide a novel model system to examine the cellular mechanisms that are required to induce Th2 responses in the intestine, and investigate whether different mechanisms exist in the small intestine and colon.

Chapter 4: IRF4$^+$ dendritic cells are required for the induction of an effective Th2 response

4.1 Results

Th2 responses are involved in many pathologies such as allergies and parasitic infections and different studies have shown the involvement of multiple cell types that can induce or maintain these responses. Both non-conventional antigen presenting cells, including basophils (Yoshimoto, 2010) and monocyte derived inflammatory dendritic cells (Plantinga et al., 2013), as well as conventional dendritic cells (DCs) (Connor et al., 2014; Phythian-Adams et al., 2010) have all been shown to play roles, either in the induction, maintenance or restimulation of the Th2 response. As we had concluded from our previous experiments that antigen is transported from the lamina propria to the MLNs, where antigen specific T cell responses were initiated, we hypothesized that conventional dendritic cells were involved. Intestinal DCs migrate through the lymphatics to the draining mesenteric lymph nodes (Cerovic et al., 2013), where they can prime naive T cells. In the small intestine and colon four different subsets of conventional dendritic cells can be identified by their differential expression of the integrins CD11b and CD103 (Persson et al., 2013a). These subsets are present at different frequencies in the small intestine and colon (Denning et al., 2011; Houston et al., 2016). Specific intestinal DC subsets are specialized to induce different facets of the T cell response. As such, CD103$^+$ DCs are able to cross present antigen to CD8$^+$ T cells and drive proinflammatory responses (Cerovic et al., 2015). CD11b$^+$ CD103$^+$ DCs are involved in the induction of Th17 responses (Persson et al., 2013b) and we reasoned that a specific DC population might also be specialized to promote Th2 responses in the intestine.

The development of both CD11b$^+$ CD103$^-$ and CD11b$^+$CD103$^+$ DC subsets is controlled by the transcription factor IRF4 (Guilliams et al., 2014) as well as local stimuli (Yokota-Nakatsuma et al., 2014). Recent studies have indeed shown that IRF4$^+$ dendritic cells are necessary for the induction of Th2 responses in the lung and skin (Gao et al., 2013; Williams et al., 2013). However, this has not been demonstrated in the gastrointestinal tract, where most parasitic Th2 responses occur.

The use of the IRF4$^{f/f}$ CD11c-cre mouse model allows the targeted deletion of IRF4 in CD11c-expressing cells, which include dendritic cells and macrophages. It has been observed that the frequency of CD11b^{+} CD103^{+} DCs is reduced in the small intestine of IRF4$^{f/f}$ CD11c-cre-positive mice (Persson et al., 2013b) but it remains unclear whether reduced cell numbers or a functional defect caused by IRF4 deletion, would impact Th2 immune responses in the intestine. We therefore addressed whether IRF4-deficiency in DCs impaired the induction of intestinal Th2 responses, and whether specific IRF4-expressing DC subsets in the small intestine and colon were developmentally or functionally affected.

4.1.1 IRF4$^{f/f}$ CD11c-cre bone marrow chimeras can be used to investigate the effects of IRF4 deficiency on dendritic cells

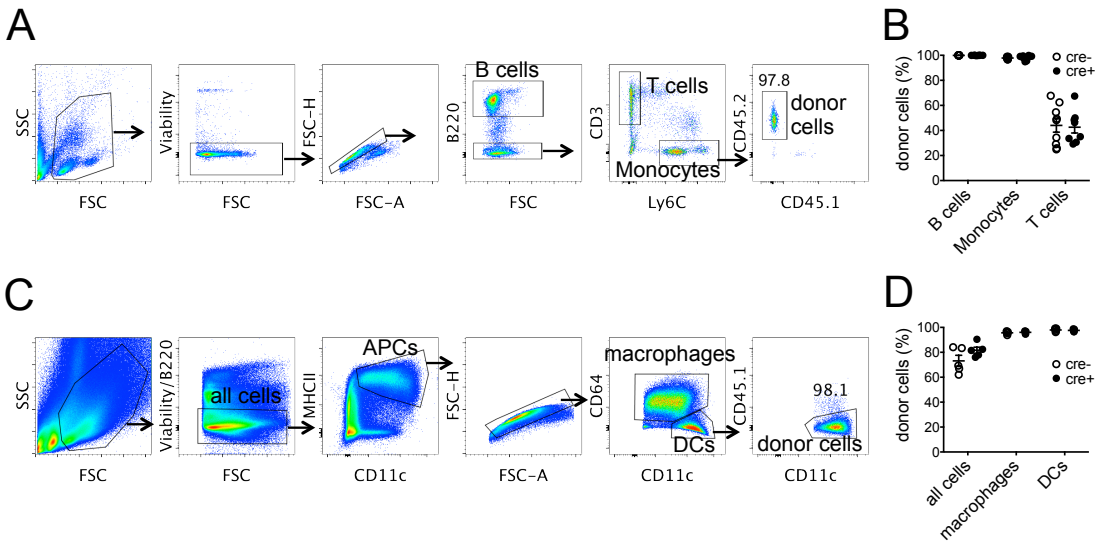

Figure 4-1. Antigen presenting cells are fully reconstituted by IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative cells in bone marrow chimeras

Cell chimerism was assessed in IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative bone marrow chimeras in blood 3 weeks after bone marrow reconstitution. The percentage of CD45.2^{+} donor cells was measured in B220^{+} B cells, Ly6C^{+} monocytes and CD3^{+} T cells by flow cytometry (A) and compared between cre+ and cre- mice (B). 6 weeks after reconstitution tissue cells were harvested. In the small intestinal lamina propria antigen presenting cells were defined as MHCII^{+} CD11c^{+} cells that could further be divided into CD64-expressing macrophages and CD11c^{hi} dendritic cells (DCs) (C). The percentage of reconstituted donor cells was again compared between cre+ and cre- mice (D). Data are representative of five independent experiments.

To address the question whether IRF4-deficiency in DCs impaired the induction of intestinal Th2 responses we generated IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeras, from bone marrow cells provided by our

collaborators Dr. Marcus Svensson-Frej and Prof. William Agace from the University of Lund, Sweden. Bone marrow chimeras were created by lethally irradiating 6-week old C57BL/6.SJL recipient mice and reconstituting them with age and sex matched 2-4 x 10^6 IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative bone marrow cells. After reconstitution, cell chimerism was monitored in blood after 3 weeks. By this time it was assumed that short-lived cells from the recipient would have been replaced by newly differentiated cells from the donor bone marrow. Blood was collected from the tail vein and red blood cells lysed using the STEMCELL EasySep™ Red Blood Cell Lysis Buffer. Cell surface markers were stained by fluorescent monoclonal antibodies and a viability dye added to exclude dead cells from analysis. Lymphocytes were distinguished from the remaining red blood cells by their larger forward scatter profile (FSC). Live single cells were separated into B cells, which express B220, T cells which were B220$^-$ Ly6C$^-$ CD3$^+$ and blood monocytes, which stained positive for Ly6C (Fig. 4-1 A). These cell populations were further analysed for their expression of CD45.2 and CD45.1. The cells that originated from the C57BL/6.SJL recipient bone marrow were CD45.1$^+$, whereas the IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative bone marrow cells were CD45.2$^+$. These distinct expression profiles allowed the separation of recipient and donor cells and allowed to assess the efficacy of bone marrow replacement. B cells and blood monocytes were almost completely replaced by cells derived from the donor bone marrow and stained for CD45.2. T cells, which are known to be more radio-resistant and long-lived, derived from donor bone marrow to around 50% (Fig. 4-1 B). No differences in cell chimerism were observed between cre+ and cre- mice and we therefore concluded that the bone marrow of all chimeras was successfully replaced, as short-lived cells that circulated in blood were completely derived from the donor bone marrow 3 weeks after reconstitution.

At the time of tissue harvest, cell chimerism was again assessed directly in the tissues. In this example, the small intestinal lamina propria was harvested 6 weeks after bone marrow reconstitution. The small intestine was dissected, cleaned, washed with HBSS/EDTA and digested in RPMI supplemented with 1 mg/ml Collagenase VIII and 10% FCS at 37° C for 15 minutes, as previously described (Houston et al., 2016). After straining, cells were stained for flow cytometry and a viability dye was added to exclude dead cells from analysis. Lymphocytes were identified by their forward and side scatter profiles and antigen presenting cells

(APCs) selected as live B220^{-} MHCII^{hi} CD11c^{+} cells. These were further divided in CD64-expressing macrophages and CD64^{-} CD11c^{hi} DCs (Fig. 4-1 C). All live cells, macrophages and DCs were further analysed for their expression of CD45.2 and CD45.1. 60-80% of all live cells were derived from the donor, indicating that certain radioresistant cells from the recipient were still present. However macrophages and dendritic cells, which we aimed to analyse and reconstitute in these chimeras, were derived from the donor bone marrow by 98% in both cre+ and cre- bone marrow chimeras (Fig. 4-1 D).

These data indicated that tissue macrophages and DCs were completely replaced by IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative cells 6 weeks after reconstitution, and that bone marrow chimeras would provide a suitable model to study the immunological effects of IRF4-deletion in these cells during *S. mansoni* egg injection.

4.1.2 Intestinal Th2 responses are impaired in IRF4$^{f/f}$ CD11c-cre-positive mice

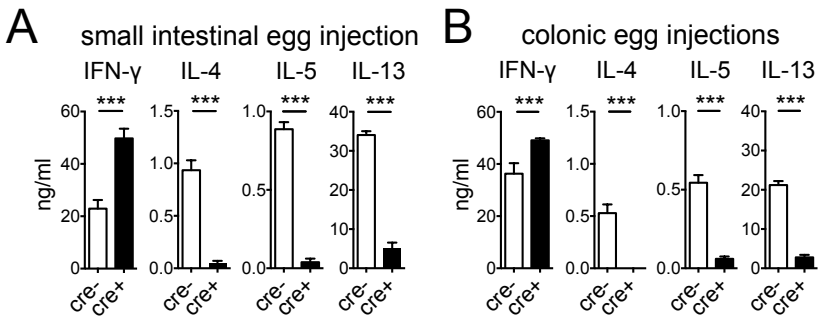

Figure 4-2. Th2 responses are impaired in the small intestine and colon of IRF4$^{f/f}$ CD11c-cre-positive mice

1,000 non-viable eggs were injected into the ileum (A) or the ascending colon (B) of IRF4$^{f/f}$ CD11c-cre-positive (cre+) or IRF4$^{f/f}$ cre-negative (cre-) mice. 5 days after immunization the individual draining lymph nodes were harvested and restimulated with SEA for 3 days. IFN-γ, IL-4, IL-5 and IL-13 were measured in the supernatants of these restimulation cultures. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (***P ≤ 0.001).

To test whether IRF4 expression by CD11c^{+} cells was necessary for the induction of Th2 responses in the intestine, we subserosally injected 1,000 non-viable *S. mansoni* eggs into the small intestines of IRF4$^{f/f}$ CD11c-cre-positive and littermate

IRF4$^{f/f}$ cre-negative bone marrow chimeric mice. As we had previously demonstrated that the ileum is drained selectively by sMLN1, this node was individually harvested 5 days after immunization. Cells were restimulated with 7.5 µg/ml SEA for 3 days. Antigen specific immune responses that had developed in response to egg injection, were assessed by measuring cytokines that were released after *in vitro* restimulation. We observed that IRF4$^{f/f}$ cre-negative mice mounted immune responses comparable to C57BL/6 mice. We detected the cytokines IFN-γ, IL-4, IL-5 and IL-13 at similar concentrations to previous experiments and measured 20 ng/ml of IFN-γ, 1 ng/ml of IL-4 and IL-5 and 30 ng/ml of IL-13 in restimulation cultures. In IRF4$^{f/f}$ CD11c-cre-positive mice IL-4, IL-5 and IL-13 were reduced to such a great extent that they were almost not detectable (Fig. 4-2 A). Levels of IFN-γ, on the other hand, more than doubled in cre-positive compared to cre-negative mice. This absence of Th2 associated cytokines and the increased production of IFN-γ indicated that Th2 responses did not develop upon small intestinal egg injection in IRF4$^{f/f}$ CD11c-cre-positive mice.
To determine whether these responses were also impaired in the colon we injected 1,000 non-viable *S. mansoni* eggs in the ascending colon. 5 days after egg injection, cMLN1 was harvested in IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative control animals and immune responses were measured after restimulation. We observed that IRF4$^{f/f}$ cre-negative mice produced antigen specific IFN-γ, IL-4, IL-5 and IL-13 upon restimulation. Similar to what we had previously observed in C57BL/6 mice, levels of Th2 cytokines were lower compared to small intestinal egg injections, with 40 ng/ml of IFN-γ, 0.5 ng/ml of IL-4 and IL-5 and 20 ng/ml of IL-13 secreted after restimulation. As observed in the small intestine, Th2 responses towards injected eggs were also affected by the deletion of IRF4 in CD11c^{+} cells in the colon, revealed by the lack of IL-4, IL-5 and IL-13 and increased levels of IFN-γ in the restimulation cultures from the cre-positive animals (Fig. 4-2 B).

We concluded that IRF4 expression by CD11c^{+} cells was essential for the effective induction of Th2 responses in the intestine, which corresponded with published findings that had shown that IRF4 expression was required in lung DCs to mount Th2 responses against house dust mite antigen (Williams et al., 2013). However, it still remained unclear if IRF4^{+} CD11c^{+} cells played a role specifically in the induction, maintenance or restimulation of the Th2 response and whether a

particular IRF4-expressing subset of cells was specialized to induce Th2 responses in the small intestine and colon.

4.1.3 IRF4 is expressed by certain subsets of dendritic cells but not by macrophages

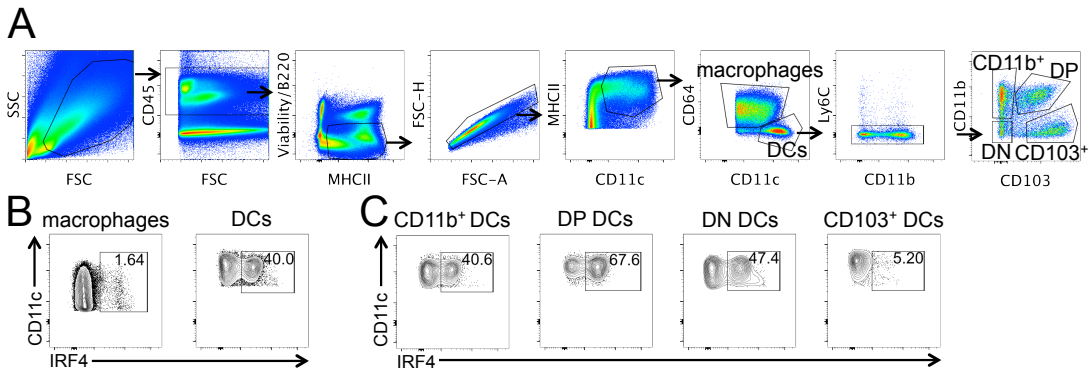

Figure 4-3. IRF4 is expressed by three intestinal DC subsets but not by macrophages or CD11b$^-$CD103$^+$ DCs

Small intestinal cells were stained for flow cytometry and CD64-expressing macrophages and CD11c^{hi} DCs identified among all MHCII$^+$ CD11c$^+$ cells. Four DC subsets were identified by their expression of CD11b and CD103 (A). This gating strategy was used for all intestinal digests. Intracellular staining showed the percentage of IRF4-expressing macrophages and DCs (B), and of each individual DC subset (C). Data are representative of three independent experiments.

Having shown that both intestinal lamina propria DCs and macrophages were replaced by IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative cells in bone marrow chimeras, we examined which CD11c$^+$ cells expressed IRF4 and would therefore be affected by a CD11c-cre-driven deletion. We harvested small intestines from C57BL/6 mice and digested them in RPMI supplemented with 1 mg/ml Collagenase VIII and 10% FCS. Cell surface markers CD45, B220, MHCII, CD11c, CD64, Ly6C, CD11b and CD103 were stained and Fixable Viability Dye eFluor780 added. Cells were fixed and permeabilized using the eBioscience Foxp3/Transcription Factor Staining Buffer Set and cells were then stained intracellularly for IRF4. Lymphocytes were selected by their forward and side scatter profile and their expression of CD45. Antigen presenting cells were selected from live single B220$^-$ cells by their expression of MHCII and CD11c. They were divided into CD64$^+$ macrophages and CD64$^-$ CD11c^{hi} Ly6C$^-$ DCs. DCs were further divided into their respective subsets by their expression of CD11b and

CD103 (Fig. 4-3 A). Four DC subsets were identified: CD11b “single positive” ($CD11b^+$) DCs, $CD11b^+$ $CD103^+$ “double positive” (DP) DCs, $CD11b^-$ $CD103^-$ “double negative” (DN) DCs and CD103 single positive ($CD103^+$) DCs.

We observed that the levels of IRF4 expression were different between intestinal macrophages and DCs. Only around 1% of macrophages expressed IRF4 whereas 40% of all DCs were $IRF4^+$ (Fig. 4-3 B). We therefore reasoned that DCs would be more affected by the deletion of IRF4 in $IRF4^{f/f}$ CD11c-cre-positive bone marrow chimeras. As DCs are also professional antigen presenting cells that migrate, transport antigen, and prime T cell responses in the draining lymph nodes, we hypothesized that the defect in Th2 induction that we had observed in $IRF4^{f/f}$ CD11c-cre-positive chimeric mice was due to IRF4-dependent effects on DCs and not macrophages.

We furthermore observed that the expression profile of IRF4 was different among the individual DC subsets. 40% of $CD11b^+$ DCs expressed IRF4; DP DCs represented the subset with the highest percentage of $IRF4^+$ cells (67%); 47% of DN DCs were $IRF4^+$ and only 5% of $CD103^+$ DCs expressed IRF4 (Fig. 4-3 C). We therefore hypothesized that $CD103^+$ DCs would be the least affected by IRF4-deletion and were the least likely subset to be involved in the induction of intestinal Th2 responses. This hypothesis was supported by reports from the literature, which demonstrated that intestinal $CD103^+$ DCs were uniquely able to cross present antigen to CD8 T cells and drive proinflammatory responses (Cerovic et al., 2015).

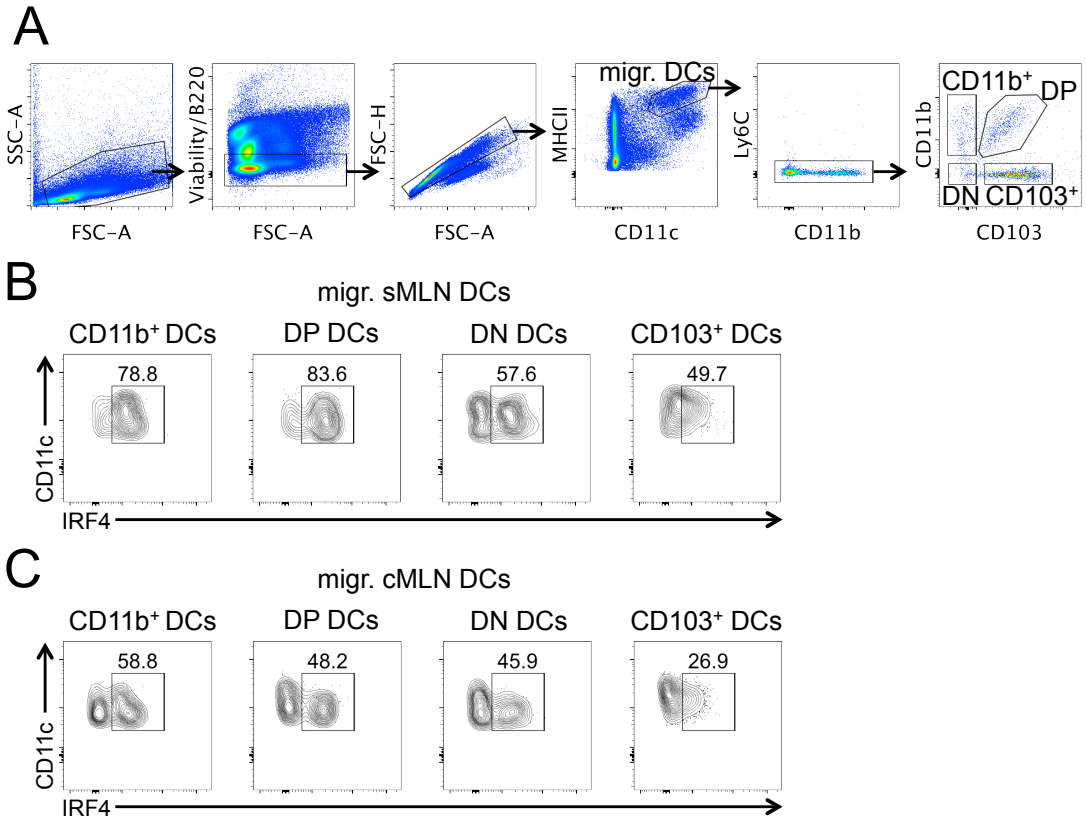

Figure 4-4. CD11b^{+} and DP DCs express the highest amount of IRF4 in small intestinal and colonic MLNs

Migratory DCs were identified as MHCII^{hi} CD11c^{+} cells in mesenteric draining lymph nodes and could be divided into four subsets based on their expression of CD11b and CD103 (A). This gating strategy was used for all MLN digests. The expression of IRF4 in CD11b^{+} and DP DCs was assessed by intracellular staining in small intestinal draining sMLNs (B) and colonic draining cMLNs (C). Expression levels were also assessed by qPCR of sorted sMLN CD11b^{+} and DP DCs using two different primer pairs (IRF4 1, and IRF4 2). The relative expression of IRF4 was normalized to DP DCs. Data are representative of three independent experiments.

It is known from the literature that DCs migrate from the intestinal lamina propria to the MLNs via the lymphatics to prime naive T cells (Cerovic et al., 2013). All four subsets of intestinal DCs migrate at steady state and are present in small intestine or colon draining MLNs at similar proportions as in the small intestinal or colonic lamina propria (Denning et al., 2011; Houston et al., 2016). These migratory DCs can be distinguished from MLN resident DCs by their higher expression of MHCII, when stained by flow cytometry. We had observed that lamina propria DC subsets expressed IRF4 to varying degrees and assessed whether migratory DCs in the MLNs expressed IRF4 to a similar extent. In order to distinguish between DCs that migrated from the small intestine or the colon, sMLNs or cMLNs of C57BL/6 mice were individually harvested. MLNs were digested as previously described (Houston et al., 2016), using RPMI supplemented with 8 U/ml Liberase and 10

µg/ml DNase for 45 minutes at 37° C. Fluorescently labelled monoclonal antibodies against the cell surface markers B220, MHCII, CD11c, Ly6C, CD11b, CD103 and Fixable Viability Dye eFluor780 were added. Cells were fixed and permeabilized using the eBioscience Foxp3/Transcription Factor Staining Buffer Set and cells were then stained intracellularly for IRF4. Migratory DCs were selected from live $B220^-$ single cells as $CD11c^+$ $MHCII^{hi}$ cells and distinguished from resident DCs by their higher expression of MHCII. $Ly6C^-$ cells were then divided into the four CD11b and CD103 expressing subsets. All four subsets could be identified and were present at similar proportions as their intestinal counterparts (Fig. 4-4 A).

In our previous experiment we had shown that IRF4 was expressed by $CD11b^+$, DP and DN DCs in the intestine but not by $CD103^+$ DCs. In sMLNs IRF4 expression was greater compared to the intestine, with 79% of $CD11b^+$ DCs, 84% of DP DCs, 58% of DN DCs and 50% of $CD103^+$ DCs expressing IRF4 (Fig. 4-4 B). Again, DP DCs represented the subset with the highest proportion of IRF4-expressing cells. However contrary to the intestine, 50% of $CD103^+$ DCs in the MLN expressed IRF4. However, the staining profile was unlike the other subsets and resembled a positive shift rather than a distinct $IRF4^+$ population. In cMLNs the proportion of IRF4-expressing migratory DCs was lower compared to the sMLNs with 59% of $CD11b^+$ DCs, 48% of DP DCs, 46% of DN DCs and 27% of $CD103^+$ DCs expressing IRF4 (Fig. 4-4 C). These data indicated that different proportions of $IRF4^+$ DCs were present in the sMLNs and cMLNs and contrary to our findings in the small intestine, certain $CD103^+$ DCs in the MLNs also upregulated their expression of IRF4. Thus, we concluded that distinct populations of $CD11b^+$, DP and DN DCs expressed IRF4 in the intestine and the draining MLNs and would likely be affected by the deletion of IRF4.

4.1.4 IRF4 affects the development of dendritic cells in the intestine but does not affect antigen uptake

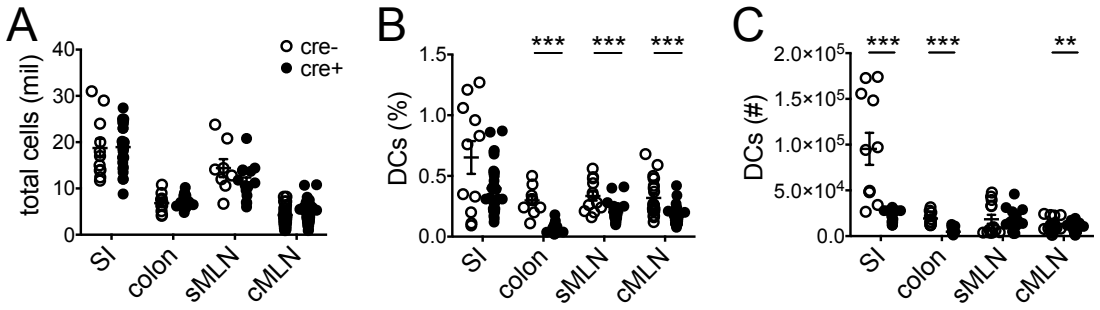

Figure 4-5. IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras manifest no changes in overall tissue cell numbers but have fewer dendritic cells

Total number of cells collected from digested small intestines (SI), colons, sMLNs and cMLNs of IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative bone marrow chimeras (A). Percentage of total DCs (B) and total number (C) after flow cytometric analysis of these tissues. Data represent at least three independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied between cre+ and cre- groups for statistical analysis (**P ≤ 0.01; ***P ≤ 0.001).

To further clarify whether the deletion of IRF4 impaired the development or the cellular functions of DCs we phenotyped the intestines and MLNs of IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative bone marrow chimeras and assessed the total number of cells and total DCs in these tissues. Small intestines, colons and MLNs were enzymatically digested and after cell straining single cell suspensions were counted and total cell numbers recorded for each tissue. As expected around 20 million cells could be isolated from the small intestine and around 7 million cells were obtained from the colon. We collected 12 million cells from sMLNs and 4 million from cMLNs. No differences in total cell numbers were observed between cre+ and cre- mice in these tissues (Fig. 4-5 A), indicating that the deletion of IRF4 in CD11c-expressing cells did not cause major disruption in the tissues, such as tissue damage or developmental defects.

We stained single cell suspensions from each tissue with fluorescent monoclonal antibodies and were able to distinguish DC populations as previously described. In the intestines total DCs were selected as live single CD45$^+$ B220$^-$ MHCII$^+$ CD11c$^+$ CD64$^-$ Ly6C$^-$ cells and in the MLNs migratory DCs were distinguished by gating on live single B220$^-$ MHCII^{hi} CD11c$^+$ Ly6C$^-$ cells. DCs accounted for around 0.5-1% of

all total cells. We had previously shown that DCs where the major IRF4-expressing $CD11c^+$ cell population in the intestine and observed a general decrease of DCs in the intestines and MLNs of $IRF4^{f/f}$ CD11c-cre-positive bone marrow chimeras. Despite not being statistically significant, the percentage of DCs tended to decrease in the small intestine and decreased to a significant level in the colon. Significant decreases were also observed in the percentage of migratory DCs in the sMLNs and cMLNs (Fig. 4-5 B). Using the total number of cells that we had counted and the respective percentage of DCs we calculated the number of DCs for each sample. Again we observed a reduction in the numbers of DCs in all tissues. Here numbers decreased significantly in the small intestine, colon and cMLN (Fig. 4-5 C).

We concluded that the deletion of IRF4 in DCs did not have a general impact on the integrity of the intestines or MLNs, as total cell numbers were comparable between $IRF4^{f/f}$ CD11c-cre-positive and $IRF4^{f/f}$ cre-negative bone marrow chimeras. However, DCs were reduced both in percentage and total number after IRF4 deletion, which suggested that IRF4 had an impact on DC development or differentiation.

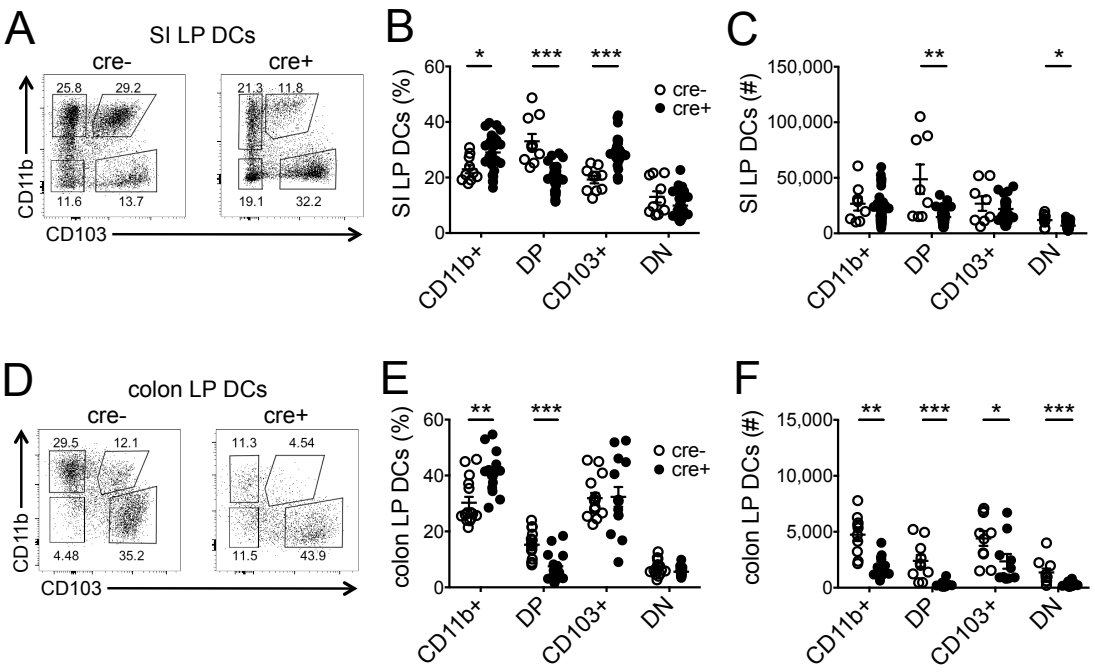

Figure 4-6. IRF4 deletion in CD11c$^+$ cells leads to a decrease in DP DCs in the small intestine and to a general reduction of DCs in the colon

DC subsets were identified by flow cytometry in small intestinal cell suspensions of IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeras (A). The percentage (B) and total number (C) of small intestinal DC subsets are displayed. DC subsets were also identified in the colon of cre+ and cre- mice (D) and their percentages (E) and total numbers (F) compared between both genotypes. Data represent at least three independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied between cre+ and cre- groups for statistical analysis (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

To assess whether the impact of IRF4 on DC development was subset specific or if it affected all DCs equally we analysed small intestinal and colonic LP DC subsets of IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeras. It has previously been reported that the deletion of IRF4 resulted in a decrease of the DP DC subset in the small intestine (Persson et al., 2013b). After digesting the small intestine with RPMI containing 1 mg/ml Collagenase VIII and 10% FCS, single cell suspensions were obtained by cell straining and stained for flow cytometry. DCs were selected as live single CD45$^+$ B220$^-$ MHCII$^+$ CD11c$^+$ CD64$^-$ Ly6C$^-$ cells and separated into four subsets by the expression of CD11b and CD103. We observed that in IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras the distribution of DC subsets was proportionally different compared to IRF4$^{f/f}$ cre-negative controls. We observed that the proportion of DP DCs decreased from 35% in cre- mice to around 20% in cre+ mice. Furthermore, we observed that CD11b$^+$ and CD103$^+$ subsets increased proportionally (Fig. 4-6 A&B). To test whether this increase of CD11b$^+$ and CD103$^+$ DCs was caused by

an increase in cell numbers, or was just a proportional increase to compensate for the decrease in DP DCs, we analysed total cell numbers. Hereby total tissue cell counts were multiplied with the percentage of total DCs and the percentage of each respective subset. We observed that the number of DP DCs decreased significantly by the deletion of IRF4. The absolute numbers of $CD11b^+$ and $CD103^+$ DCs however were not affected (Fig. 4-6 C). We concluded that the DP DC subset was the only subset in the small intestinal lamina propria that was affected by the deletion of IRF4 and decreased in number, confirming already published findings (Persson et al., 2013b).

However, it remained unknown how the deletion of IRF4 in CD11c cells affected the composition of DC subsets in the colonic lamina propria. We harvested the colons of $IRF4^{f/f}$ CD11c-cre-positive and $IRF4^{f/f}$ cre-negative bone marrow chimeric mice and digested them enzymatically. Cells were stained with fluorescent monoclonal antibodies and DC subsets identified as CD11b/CD103 expressing live single $CD45^+$ $B220^-$ $MHCII^+$ $CD11c^+$ $CD64^-$ $Ly6C^-$ cells. Whereas DP and $CD103^+$ DCs comprised the majority of DCs in the small intestine, $CD11b^+$ and $CD103^+$ DCs were the most abundant subsets in the colon. We observed that the proportion of DC subsets was different in cre+ mice, with an increase of $CD11b^+$ DCs from 30% to 40% and a reciprocal decrease of DP DCs from 15% to 5% (Fig. 4-6 D&E). The proportion of $CD103^+$ and DN DCs was not statistically affected but $CD103^+$ were slightly increased. These proportional changes were similar to our observations in the small intestine and showed a decrease of DP DCs and a reciprocal increase of $CD11b^+$ and $CD103^+$ DCs.

However, the analysis of total cell numbers revealed that only DP DCs were decreased in the small intestine. When we analysed total cell numbers of colonic DCs we observed that they were generally 10-fold lower compared to the small intestine, due to a lower percentage of DCs and smaller tissue size. This difference had been observed in C57BL/6, cre+ and cre- mice and has also been reported in the literature (Denning et al., 2011; Houston et al., 2016). Comparing total cell numbers between $IRF4^{f/f}$ CD11c-cre-positive and $IRF4^{f/f}$ cre-negative mice in the colon, we noticed that all DC subsets were reduced. Hereby, DP DCs were the most affected population, whereas $CD103^+$ DCs showed the least decrease in cell numbers (Fig. 4-6 F). This suggested that IRF4 deletion had a general developmental effect on colonic DCs and decreased their number in the

lamina propria. Overall, DP DCs were the most affected subset in both the small intestine and colon, whereas CD103^{+} DCs were the least affected. We therefore hypothesized that DP DCs were important for the induction of Th2 responses and that their decrease was the cause for the defective Th2 response in IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras after egg injection.

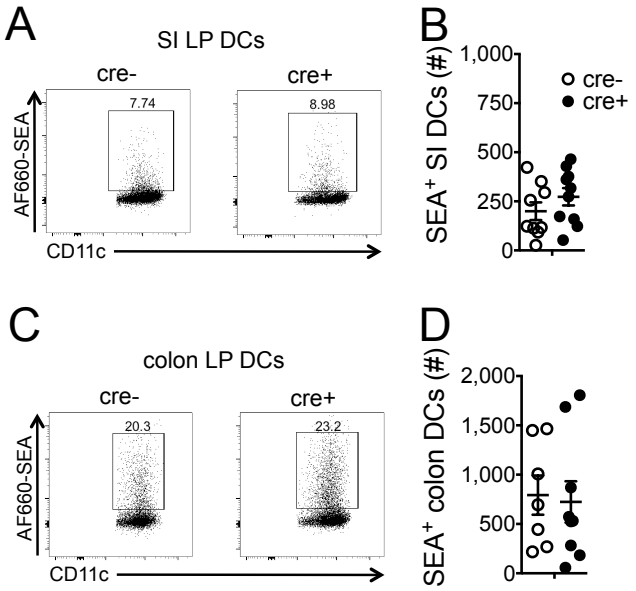

Figure 4-7. Antigen uptake by intestinal DCs is not affected by the deletion of IRF4

20 µg of AlexaFluor660-labled SEA was injected into the small intestinal lamina propria of IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeras. Levels of SEA uptake by DCs were measured 24 hours after injection (A). Total numbers of AF660-SEA^{+} SI LP DCs were compared between cre+ and cre- mice (B). SEA-AF660 was also injected into the colonic lamina propria and uptake by LP DCs assessed after 24 hours (C). Total numbers of AF660-SEA^{+} colon LP DCs were compared between cre+ and cre- mice (D). Data represent at least three independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied between cre+ and cre- groups for statistical analysis.

As we hypothesised that the decrease of DP DCs in the small intestinal lamina propria and the colon affected the induction of Th2 responses we tested whether antigen uptake by lamina propria DCs was adversely affected in IRF4$^{f/f}$ CD11c-cre-positive mice. We fluorescently labelled soluble egg antigen with AlexaFluor660 and injected 20 µg into the small intestinal lamina propria of IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeric mice. From previous experiments we knew that this amount was equivalent to amounts used to drive robust Th2 responses, which suggested that it was likely to be taken up by DCs in the lamina propria and transported to the draining lymph nodes. 24 hours

after AF660-SEA injection the injected area of the small intestine was excised and digested with Collagenase VIII. Cells were stained with fluorescent monoclonal antibodies and analysed by flow cytometry. We gated on total lamina propria DCs and analysed the level of AF660 fluorescence as a measure of antigen uptake. We observed that around 8% of all DCs labelled positive for AF660-SEA in both cre+ and cre- mice (Fig. 4-7 A). This suggested that despite the change in DC subset composition in cre+ mice uptake of parasite antigen was not affected in the lamina propria. This was confirmed by comparing the total number of SEA-labelled DCs in the small intestine where also no difference between cre+ and cre- mice was observed (Fig. 4-7 B).

We had observed that IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras had generally fewer colonic DCs compared to their IRF4$^{f/f}$ cre-negative littermates, with DP DCs being the most affected subset. To test whether this decrease in DC numbers affected the uptake of parasite antigen in the colon, we injected 20 µg AF660-SEA in the colonic lamina propria. 24 hours after AF660-SEA injection, colons were harvested from injected IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeras and digested with Collagenase V, Collagenase D, Dispase and DNase. Cells were stained for flow cytometry and levels of AF660 measured to indicate AF660-SEA uptake. We gated on total colonic DCs and observed that around 20% of cre+ and cre- cells had taken up AF660-SEA and that the total number of AF660-SEA^{+} colonic DCs was comparable between cre+ and cre- mice (Fig. 4-7 C&D). This again suggested that uptake of antigen was not affected in IRF4$^{f/f}$ CD11c-cre-positive mice despite the fact that numbers of DCs were reduced in these mice. Taken together, we had observed no difference in the uptake of AF660-labeled SEA by small intestinal and colonic DCs in IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative mice. This suggested that IRF4-deficiency and the decrease of DP DCs in the lamina propria of cre+ mice did not have an impact on antigen uptake and could not explain the impairment of Th2 responses in these mice.

4.1.5 CD11b-expressing dendritic cells are dramatically reduced in the MLNs of IRF4$^{f/f}$ CD11c-cre-positive mice, which affects antigen availability

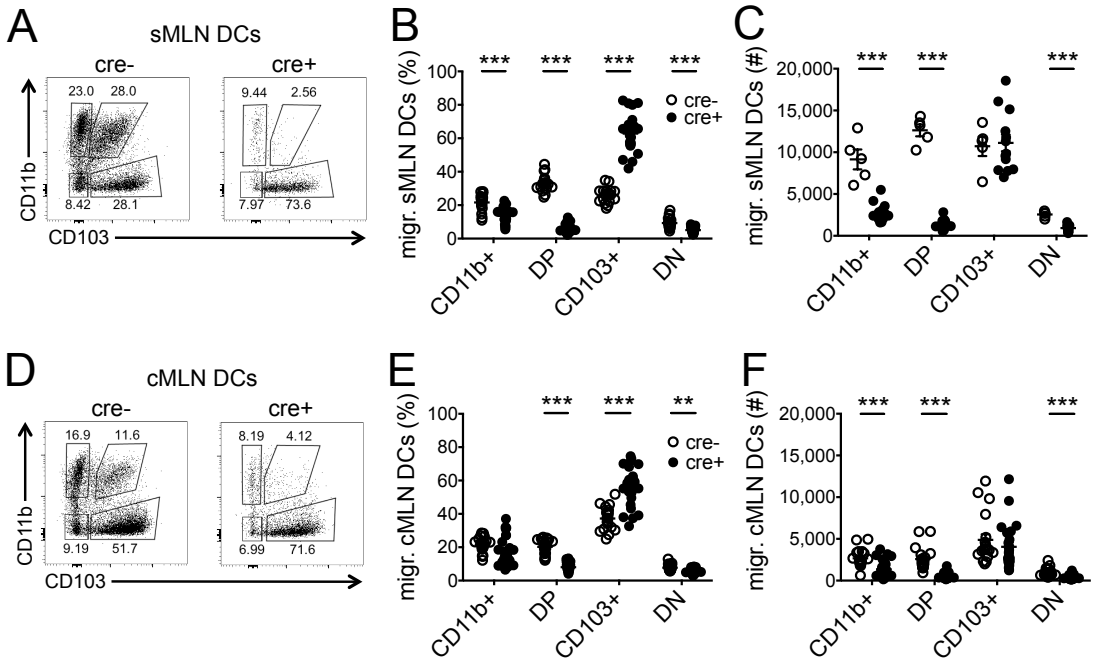

Figure 4-8. Fewer CD11b^{+} and DP migratory DCs are present in the sMLNs and cMLNs of IRF4$^{f/f}$ CD11c-cre-positive mice

Small intestinal draining MLNs were harvested from IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeras, digested and analysed by flow cytometry (A). The percentage (B) and total number (C) of migratory DC subsets was compared between cre+ and cre- mice. Migratory cMLN DCs were also assessed by flow cytometry (D) and percentages (E) and total numbers (F) compared between cre+ and cre- mice. Data represent at least three independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied between cre+ and cre- groups for statistical analysis (**P ≤ 0.01; ***P ≤ 0.001).

In addition to an intestinal phenotype it has also been reported that IRF4-deletion affected DC migration in the skin (Bajaña et al., 2012) and reduced numbers of CD11b^{+} and DP DCs have also been observed in the MLNs of IRF4$^{f/f}$ CD11c-cre-positive mice (Persson et al., 2013b). We therefore investigated if the deletion of IRF4 also had an impact on DC subsets in the small intestinal and colonic draining MLNs. We harvested the small intestinal draining sMLNs from IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeras and digested them with 8 U/ml Liberase and 10 µg/ml DNase in RPMI. Single cell suspensions were stained with fluorescent monoclonal antibodies against the cell surface markers B220, MHCII, CD11c, Ly6C, CD11b and CD103. Migratory DCs that expressed higher levels of MHCII compared to resident DCs, were divided into four subsets by their

differential expression of CD11b and CD103. All four subset were clearly distinguishable and $CD11b^+$, DP and $CD103^+$ were present in equal proportions in cre- mice. In $IRF4^{f/f}$ CD11c-cre-positive mice we observed that $CD11b^+$ DCs decreased by 50% compared to cre-negative littermates. Furthermore, DP DCs decreased by over 90%, making this population hardly detectable. Reciprocally the proportion of $CD103^+$ DCs increased from 30% to 70% (Fig. 4-8 A&B). The decrease of $CD11b^+$ and DP DCs was also observed when we compared total numbers of DCs. The number of $CD11b^+$ DCs dropped from 10,000 in cre- mice to 2,000 in cre+ mice and DP DCs were even further reduced and decreased from 13,000 to 1,000 cells. The number of $CD103^+$ DCs was however not affected by the deletion of IRF4, an observation that we had also made in the small intestinal lamina propria (Fig. 4-8 C). However, contrary to our findings in the lamina propria, where only DP DCs were decreased, sMLNs showed a more pronounced effect. $CD11b^+$ DCs were reduced by 50% and DP DCs were hardly present in $IRF4^{f/f}$ CD11c-cre-positive mice, suggesting that changes in DC proportions in the MLNs rather than the small intestine might contribute to the observed defects of Th2 immunity.

As the deletion of IRF4 also affected DCs in the colonic lamina propria, by reducing the number of all DC subsets in particular of DP DCs, we hypothesised that migratory DCs in the colon draining cMLNs would also be affected. We harvested cMLNs from $IRF4^{f/f}$ CD11c-cre-positive and $IRF4^{f/f}$ cre-negative bone marrow chimeric mice and digested them with Liberase and DNase. Cells were stained with fluorescent antibodies and analysed by flow cytometry. We observed that the general distribution of DC subsets was different to sMLNs. Whereas $CD11b^+$, DP and $CD103^+$ DCs were present at similar proportions in the sMLNs, $CD103^+$ DCs were the most abundant subset in cMLNs. The deletion of IRF4 had a similar effect in cMLNs as it had in sMLNs reducing $CD11b^+$ and DP DCs and increasing the proportion of $CD103^+$ DCs. However the decrease of $CD11b^+$ DCs was not statistically significant and the reduction of DP DCs was not as pronounced but decreased from 20% in cre- mice to 5% in cre+ mice (Fig. 4-8 D&E). The total number of each DC subset in the cMLN followed a similar trend as we had already observed in sMLNs. The number of $CD103^+$ DCs was not affected by the deletion of IRF4, whereas the number of $CD11b^+$ DCs was reduced and the number of DP DCs even more affected (Fig. 4-8 F).

We therefore observed that the deletion of IRF4 not only affected DC subsets in the lamina propria of the small intestine and colon, where we had observed the most pronounced reduction in DP DCs, but also reduced DC subsets in the MLNs. In both small intestinal and colonic draining MLNs CD11b^{+} DCs were reduced by 50% and DP DCs were hardly present. We therefore hypothesized that the pronounced decrease of MLN CD11b^{+} and DP DCs in IRF4$^{f/f}$ CD11c-cre-positive mice affected T cell priming, which could lead to the impairment of Th2 immunity observed in these mice.

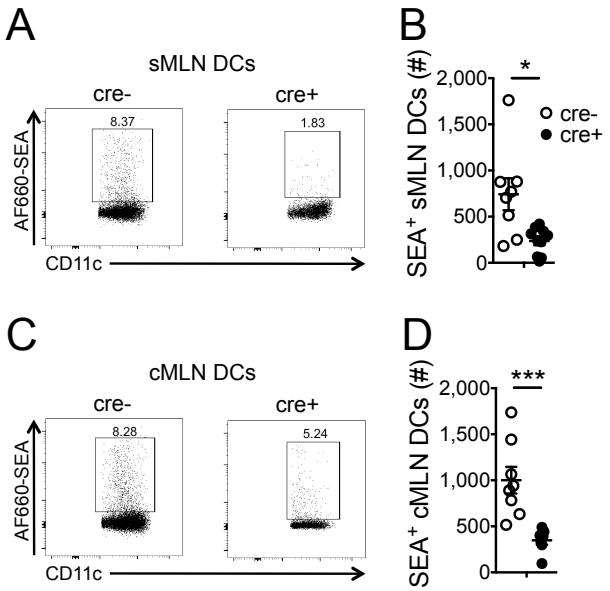

Figure 4-9. IRF4 deletion in CD11c^{+} cells reduces the number of antigen-carrying DCs in the MLNs

Antigen-carrying migratory DCs were visualized by the injection of 20 µg SEA-AF660 into the small intestinal lamina propria. 24 hours after injection the sMLN that drained the injection site was harvested, digested and cells stained for flow cytometry (A). Total numbers of AF660-SEA^{+} migratory sMLN DCs were compared between cre+ and cre- mice (B). SEA-AF660 was also injected into the colonic lamina propria and transport to the cMLNs assessed after 24 hours (C). Total numbers of AF660-SEA^{+} migratory cMLN DCs were compared between cre+ and cre- mice (D). Data represent at least three independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied between cre+ and cre- groups for statistical analysis (*P ≤ 0.05; ***P ≤ 0.001).

We had observed that antigen uptake by lamina propria DCs was not affected by IRF4-deficiency. However, we hypothesized that the severe reduction of CD11b^{+} and DP DCs in the MLNs would affect the number of antigen-carrying DCs in IRF4$^{f/f}$ CD11c-cre-positive mice. To test this hypothesis, we injected 20 µg AF660-labelled SEA into the small intestinal lamina propria of IRF4$^{f/f}$ CD11c-cre-positive

and IRF4$^{f/f}$ cre-negative bone marrow chimeras. 24 hours after injection the individual sMLN that drained the injection site was harvested and digested with 8 U/ml Liberase and 10 µg/ml DNase. Cells were stained for flow cytometry and migratory DCs selected as live single B220^{-} MHCII^{hi} CD11c^{+} Ly6C^{-} cells. DCs that labelled positive for AF660-SEA were considered to be antigen-transporting cells that had taken up AF660-SEA from the lamina propria and migrated through the lymphatics to the draining lymph nodes. We observed that 8% of DCs had taken up antigen in cre- mice but only 1% of DCs were AF660-SEA^{+} in cre+ animals (Fig. 4-9 A). We observed a similar reduction comparing total numbers of AF660-SEA^{+} DCs where an average of 800 cells was present in cre- sMLNs but only 200 AF660-SEA^{+} DCs were collected from cre+ sMLNs (Fig. 4-9 B). This observation supported our hypothesis that the reduced number of CD11b^{+} and DP DCs in the sMLNs of IRF4$^{f/f}$ CD11c-cre-positive mice resulted in decreased numbers of antigen-carrying DCs. This reduction in cell numbers and available antigen in the MLNs would likely affect T cell priming and could explain the impaired induction of Th2 responses in these mice.

As we had observed a similar reduction of CD11b^{+} and DP DCs in the colonic draining lymph nodes we expected a comparable decrease of antigen-carrying DCs in the cMLNs. We injected AF660-SEA in the colonic lamina propria of IRF4$^{f/f}$ CD11c-cre-positive and IRF4$^{f/f}$ cre-negative bone marrow chimeric mice. Colon draining cMLNs were harvested 24 hours after injection and digested using Liberase and DNase. Cells were stained for flow cytometry and the levels of AF660 fluorescence analysed on migratory DCs. Similar to our observations in sMLNs, 8% of cMLN DCs were AF660-SEA^{+} in cre- mice, which was equivalent to 1,000 cells, whereas in cre+ mice the percentage of AF660-SEA^{+} DCs was reduced and only 300 DCs labelled positive for AF660-SEA (Fig. 4-9 C&D). These results were comparable to what we had observed in the small intestinal draining MLNs and showed that fewer antigen-carrying migratory DCs were present in the MLNs of IRF4$^{f/f}$ CD11c-cre-positive mice. DC migration as well as survival have been suggested in the literature to contribute to the reduction of migratory CD11b-expressing DCs in the draining lymph nodes (Bajaña et al., 2012; Schlitzer et al., 2013; Persson et al., 2013b). However, no direct consequence for antigen availability had been demonstrated in these studies. We concluded that the decrease of CD11b^{+} and DP migratory DCs in the MLNs of cre+ mice and the concurrent reduction of transported antigen could impact T cell priming and

contribute to the impaired T cell responses observed in IRF4$^{f/f}$ CD11c-cre-positive mice.

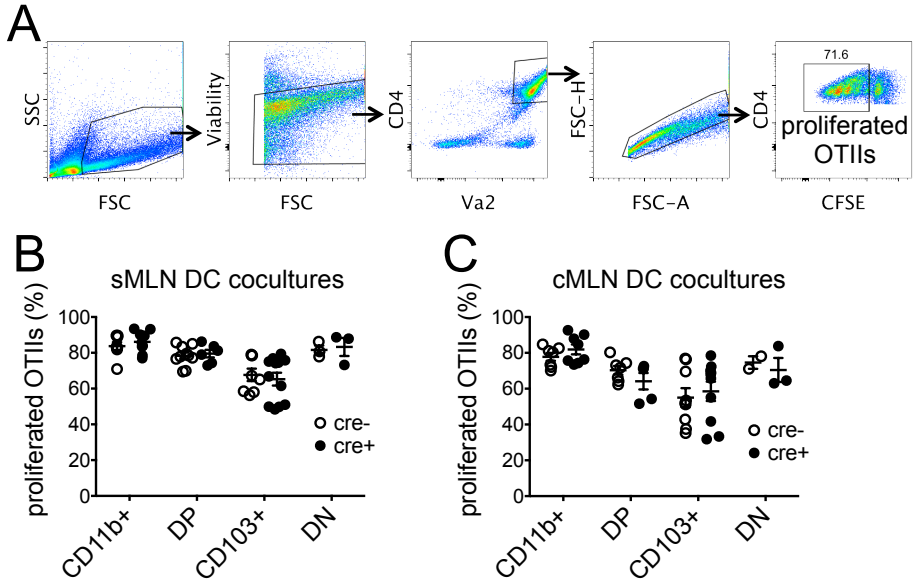

Figure 4-10. Antigen presentation is not affected on a cell-to-cell basis in IRF4$^{f/f}$ CD11c-cre-positive MLN DCs

Antigen presentation by individually FACS purified MLN DC subsets was assessed in OVA/OT-II cocultures with CFSE-labelled OT-II MLN cells. After 3 days of incubation cocultures were stained with anti-CD4, anti-Va2 and a viability dye. Proliferated CD4 OT-II T cells were identified by their reduced concentration of CFSE (A). The level of proliferation induced by 6,000 migratory sMLN DCs (B) or 3,000 migratory cMLN DCs (C) from IRF4$^{f/f}$ CD11c-cre-positive or IRF4$^{f/f}$ cre-negative mice was compared. Data represent at least three independent experiments (mean ± SEM) with each point representing one coculture. Mann-Whitney *U* tests were applied between cre+ and cre- groups for statistical analysis.

To demonstrate that reduced numbers of CD11b^{+} and DP migratory DCs in the MLNs and the concurrent reduction of transported antigen, and not functional defects in T cell priming, contributed to the impaired Th2 induction in IRF4$^{f/f}$ CD11c-cre-positive mice, we assessed T cell priming capabilities by DCs *in vitro.*

To assess if IRF4-deficiency in DCs affected antigen presentation and T cell priming we used OVA/OT-II *in vitro* cocultures to monitor T cell priming by DCs. Transgenic OT-II mice have been genetically modified so that CD4 T cells only express a specific T cell receptor especially designed to recognize OVA peptide (Robertson et al., 2000). Upon presentation of OVA by antigen presenting cells CD4 T cells proliferate and T cell priming can thus be selectively monitored. To test whether IRF4$^{f/f}$ CD11c-cre-positive DCs were impaired in processing and

presenting antigen, we harvested small intestinal draining lymph nodes and digested them with Liberase and DNase. Cre+ and cre- cells were stained with monoclonal fluorescent antibodies and DC subsets were individually sorted by FACS and cells were loaded with 2 mg/ml OVA protein *in vitro*. After two hours, cells were washed thoroughly and 6,000 DCs of each subset from cre+ or cre- mice were incubated with 200,000 CFSE-labelled MLN cells from OT-II mice. After 3 days of coculture, cells were stained with anti-CD4, anti-Vα2 and a cell viability dye. Cells were analysed by flow cytometry and OT-II CD4 T cells selected by their expression of CD4 and the TCR Vα2 (Fig. 4-10 A) (Leung et al., 2013). Proliferated T cells were assessed by their reduced signal of CFSE. This fluorescent molecule is taken up by cells and remains within the cytosol where it is distributed between both daughter cells during cell division. Thus, reduced levels of fluorescence mark proliferated cells which contain lower concentrations of CFSE compared to undivided cells (Quah et al., 2007). We observed that roughly 80% of all OT-II CD4 T cells proliferated after coculture with OVA-loaded DCs and $CD103^+$ DCs induced the lowest amount of proliferation of around 70%. We did not observe any difference in OTII proliferation between cre+ and cre- DC cocultures, suggesting that IRF4-deficient DCs were as efficient in priming naive T cells as equal numbers of $IRF4^{f/f}$ cre-negative DCs (Fig. 4-10 B). This suggested that antigen presentation and T cell priming were not affected by the absence of IRF4 in small intestinal MLN DCs.

As IRF4-deletion also reduced numbers of $CD11b^+$ and DP migratory DCs as well as the number of antigen-carrying DCs in the cMLNs, we assessed the impact of IRF4-deficiency on antigen presentation and T cell priming by migratory cMLN DCs. As DCs in the cMLN were less abundant, only 3,000 DCs from each subset were FACS sorted from $IRF4^{f/f}$ CD11c-cre-positive and $IRF4^{f/f}$ cre-negative mice and incubated with 2 mg/ml OVA protein for two hours. DCs were cocultured with 200,000 CFSE labelled MLN cells from OTII mice, and after 3 days T cell proliferation was assessed by flow cytometry. Again, we observed that around 80% of CD4 OTII T cells proliferated after coculture with each individual cMLN DC subset. $CD103^+$ DCs were the least effective in priming CD4 OTII T cells with around 60% proliferation and no difference in proliferation of CD4 OTII T cells cocultured with cre+ or cre- DC subsets was observed (Fig. 4-10 C). This confirmed that IRF4-deficiency did not impair antigen presentation or priming of CD4 OTII T cells by small intestinal and colonic MLN DCs but rather indicated that

the reduced number of antigen-carrying migratory DCs in cre+ mice affected the induction of T cell responses in these mice.

4.2 Discussion

It has been shown in the literature that the transcription factor IRF4 controls type 2 immune responses in a number of cell types. IRF4 directly controls Th2 cell differentiation, alternative activation of macrophages and dendritic cell development. Furthermore, IRF4 has also been suggested to control functional mechanisms in DCs that specifically enable them to drive Th2 responses.

Using the IRF4$^{f/f}$ CD11c-cre chimeric mouse model we were able to address the IRF4 dependency of CD11c^{+} cell development and its necessity for the adequate induction of Th2 responses in the intestine. It has previously been reported that IRF4^{+} CD11c^{+} cells are specialized to drive Th2 responses against house dust mite in the lung, which did not develop in IRF4$^{f/f}$ CD11c-cre-positive mice (Williams et al., 2013). We observed a similar effect when we injected *S. mansoni* eggs into the intestines of IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras and observed that Th2 responses were impaired and IFN-γ responses were increased in MLN restimulation cultures (Fig. 4-2). We had previously observed a similar reciprocal increase in IFN-γ responses in IL-4$^{-/-}$ mice, which indicated that the proinflammatory responses against egg antigens are dampened by type 2 immune responses and are increased in their absence. The observation that proinflammatory responses were not reduced by the deletion of IRF4 in DCs has also previously been observed in cytotoxic T lymphocyte responses during allergic airway inflammation (Vander Lugt et al., 2014), suggesting that IRF4 deletion specifically affects the induction of type 2 immune responses. Therefore, our findings corresponded with the literature and confirmed an IRF4 dependency of CD11c^{+} cells in inducing efficient Th2 responses. In fact, Th2 responses in the small intestine as well as the colon were impaired, suggesting a common underlying mechanism.

To assess which antigen presenting cells expressed IRF4, and were therefore candidates for priming Th2 cells, we performed intracellular staining on intestinal CD11c^{+} cells. We observed that DCs but not macrophages expressed IRF4. As DCs are specialized in the uptake of tissue antigen, and migrate to the draining lymph nodes to induce T cell responses, it is likely that they are also involved in priming Th2 responses in the intestine. The expression of IRF4 was variable among DC subsets and was expressed by intestinal CD11b^{+}, DP and DN DCs but

not by $CD103^+$ DCs in the intestine (Fig. 4-3). Hereby, DP DCs represented the subset with the highest proportion of $IRF4^+$ cells, suggesting that they might be most affected by the deletion of IRF4. To understand whether IRF4 affected the development of DCs, we harvested intestines and MLNs from $IRF4^{f/f}$ CD11c-cre+ and cre- bone marrow chimeras. We observed that total cell numbers in the intestines and MLNs were not affected by the deletion of IRF4 in CD11c-expressing cells, indicating that this deletion did not cause major disruption in the tissues, such as tissue damage or developmental defects. However, the percentage and number of DCs was affected in all tissues (Fig. 4-5). More detailed analysis showed that the number of DP DCs was reduced in the small intestine and that all DCs were reduced in the colon, with DP DCs being the most affected colonic DC subset (Fig. 4-6). Consistently, a reduction in small intestinal DP DCs has previously been reported in $IRF4^{f/f}$ CD11c-cre-positive mice (Persson et al., 2013b). The reduction of DC numbers indicated that IRF4 was involved in the development of DCs and, consistent with its highest expression in DP DCs, mostly affected this subset in the intestine. However, uptake of fluorescently labelled parasite antigen by DCs in the intestine was not affected in $IRF4^{f/f}$ CD11c-cre-positive bone marrow chimeras (Fig. 4-7), suggesting that the function of IRF4-deficient DCs was not impaired.

Analysis of DC subsets in the small intestinal and colonic MLNs revealed an even more striking reduction of $CD11b^+$ DCs and a near absence of DP DCs, whereas $CD103^+$ DCs were not affected (Fig. 4-8). A similar reduction of $CD11b^+$ and DP DCs in the MLNs has also been observed in the literature (Persson et al., 2013b), although it remains unclear if defects in migration, development or cell survival, which have all been suggested (Schlitzer et al., 2013; Persson et al., 2013b; Bajaña et al., 2012), affected migratory DCs in the MLNs of $IRF4^{f/f}$ CD11c-cre-positive mice. Concurrently, the number of antigen-carrying DCs was also reduced in the MLNs (Fig. 4-9), suggesting that reduced numbers of antigen-presenting DCs and available antigen in the MLNs led to the inefficient priming of Th2 responses in $IRF4^{f/f}$ CD11c-cre-positive mice. This hypothesis was further strengthened by our observation that the remaining IRF4-deficient DCs were as efficient as cre-negative DCs in presenting OVA protein and priming OT-II T cells in *in vitro* cocultures, which showed that they were not functionally impaired (Fig. 4-10). However, further experiments are needed to verify that IRF4-deficiency in DCs does not specifically inhibit their capacity to induce Th2 responses, as has

been suggested by Williams *et al.*, who have shown that IRF4 directly regulates IL-10 and IL-33 expression in DCs (Williams et al., 2013).

From the low expression of IRF4 on $CD103^+$ DCs, their unchanged presence in the intestines and MLNs of $IRF4^{f/f}$ CD11c-cre-positive mice and published findings that indicate that intestinal $CD103^+$ DCs are specialized to cross present antigen to CD8 T cells and elicit proinflammatory responses (Cerovic et al., 2015), we considered it likely that intestinal $CD103^+$ DCs were not involved in the induction of Th2 responses. From the literature as well as our own observations, CD11b-expressing DCs were the most likely candidates. However, as both $CD11b^+$ and DP DCs were reduced in the MLNs of $IRF4^{f/f}$ CD11c-cre-positive mice, which is the tissue where T cell priming occurs, it remained unclear which subset was specialized to induce Th2 responses in the intestine. We therefore continued our research using C57BL/6 mice and separately assessed the involvement of the different DC subsets in the induction of Th2 responses.

4.3 Conclusions

We confirmed in our intestinal *S. mansoni* egg injection model that Th2 responses were impaired if CD11c-expressing cells did not express IRF4. IRF4 was predominantly expressed by intestinal DCs but not by macrophages. We observed changes in the composition of lamina propria and MLN DC subsets in $IRF4^{f/f}$ CD11c-cre-positive bone marrow chimeras, where CD11c cells were fully replaced in blood and tissues after 3 and 6 weeks. The number of DP DCs was greatly reduced in the small intestine. In the colon all DC subsets were reduced, but DP DCs were again the most affected DC subset. However, these changes did not interfere with the uptake of AlexaFluor660-labeled SEA, and no difference between the numbers of AF660-SEA^+ DCs was observed in the small intestinal or colonic lamina propria of $IRF4^{f/f}$ CD11c-cre-positive and $IRF4^{f/f}$ cre-negative mice. Consistent with published findings, migratory DC subsets in the MLN were greatly affected by the deletion of IRF4. Here, $CD11b^+$ DCs were reduced by 50% and DP DCs were almost completely absent in small intestinal and colonic draining MLNs, leading to a significant decrease in antigen-carrying cells. Antigen presentation and T cell priming capabilities were however not impaired in cre+ mice, when equal numbers of DCs were loaded with OVA and cocultured with CFSE-labelled MLN cells from OT-II mice. We therefore concluded that it was not functional defects of IRF4-deficient DCs that influenced the induction of Th2 responses against *S. mansoni* eggs. Instead, we suggest that the reduction of $CD11b^+$ and DP DCs in the MLNs and the concurrent decrease in antigen-carrying cells led to the impaired Th2 response in $IRF4^{f/f}$ CD11c-cre-positive mice, although specific Th2-inducing defects by IRF4-deletion have to be considered and need further investigation.

Chapter 5: Characterization of lymph migratory immune cells after intestinal injection of *Schistosoma mansoni* eggs

5.1 Results

Our observations in IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras showed that the numbers of CD11b-expressing DC subsets were reduced in the MLNs, which impaired transport of egg antigen to the MLNs and likely affected the induction of Th2 responses against *S. mansoni* eggs. These DCs must have migrated from the lamina propria through the lymphatics to reach the individual draining MLNs. In order to assess the effects of *S. mansoni* egg injection on migratory intestinal DCs we directly analysed these cells in lymph. Thoracic duct lymph was collected from mesenteric lymphadenectomized mice by the surgical insertion of a cannula (Cerovic et al., 2013). To separately assess if specific DC subsets were specialized to transport parasite antigen from the small intestine and colon, either the sMLNs or cMLNs were removed prior to thoracic duct cannulation. Following removal of sMLNs ("sMLNx"), DCs in thoracic duct lymph were small-intestine-derived, whereas cMLN removal ("cMLNx") enabled colonic DCs to migrate to the thoracic duct (Houston et al., 2016).

5.1.1 Thoracic duct cannulations allow the collection of intestinal lymph

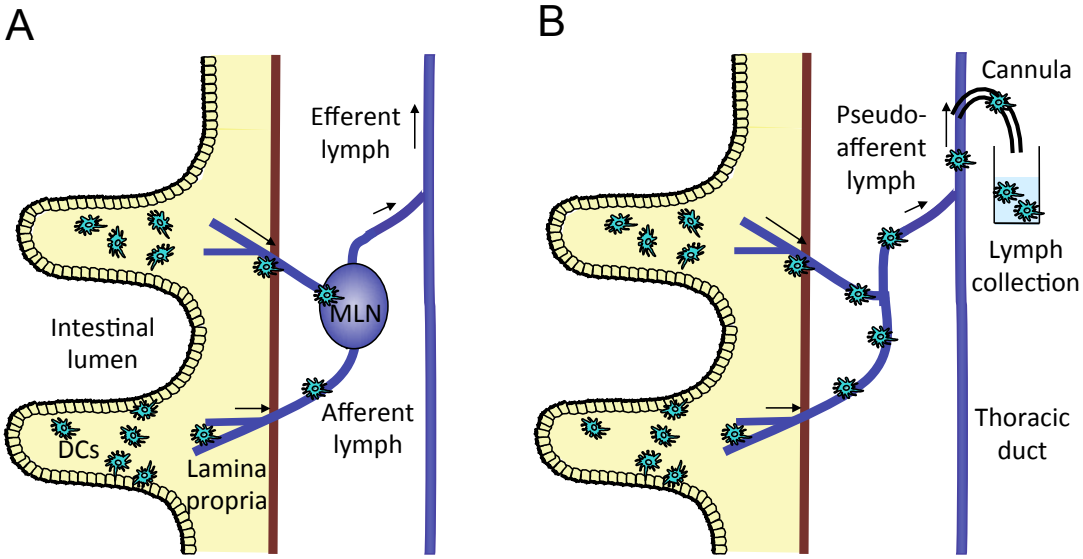

Figure 5-1. Cannulation of lymphadenectomized mice allows the collection of DCs in thoracic duct lymph

Schematic illustrating the flow of lymph and migration of DCs from the intestinal lamina propria to the MLNs (A). Surgical removal of the MLNs allowed DC migration to the thoracic duct where lymph was collected by cannulation (B).

Thoracic duct cannulations are based on the principle that lymph flows from the intestinal lamina propria to the MLNs as afferent lymph, transporting cells and soluble factors. Lymph exits the MLN through the efferent lymphatics and feeds into the thoracic duct. We have developed a surgical method that allows the insertion of a polyurethane medical grade intravascular tube into the thoracic duct. After surgical recovery, lymph can thus be collected for up to 18 hours. Analysis of lymph from cannulated C57BL/6 mice has identified immune cell populations such as B cells and T cells. However, dendritic cells were not identified as they do not exit the MLNs to reach the thoracic duct (Fig. 5-1 A). Surgical removal of the MLNs 6 weeks prior to cannulation enables the collection of “pseudoafferent” lymph. Following the removal of the lymph nodes, the afferent and efferent lymphatics reanastamose, which allows the collection of DCs in thoracic duct lymph (Fig. 5-1 B). The fact that DCs cannot migrate beyond the draining lymph nodes allows further anatomical manipulation. By selectively removing the sMLNs, only small intestinal draining DCs will reach the thoracic duct, whereas removing

the cMLNs ensures that only colonic DCs are collected (Houston et al., 2016). It is thus possible to separately assess lymph migratory DCs from the small intestine or colon.

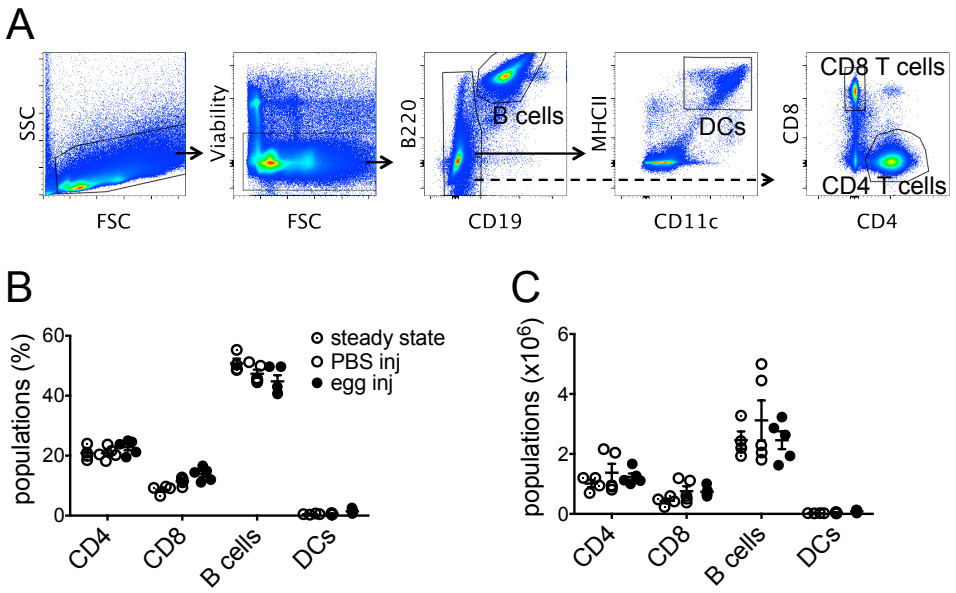

Figure 5-2. Intestinal egg injections do not significantly affect the migration of B cells, T cells and total DCs in lymph

B cells, DCs and CD4 and CD8 T cells were analysed in lymph from cannulated MLNx mice following the presented gating strategy (A). The percentage (B) and total number (C) of these immune cells were compared between uninjected (steady state), PBS and egg injected mice. Data represent two independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied between PBS injected and egg injected groups for statistical analysis.

To address if the injection of material into the intestine, particularly of *S. mansoni* eggs, affected the migration of immune cells in lymph, we analysed thoracic duct lymph from uninjected, PBS injected and egg injected animals. Total MLNx mice were cannulated, 20 µl PBS or 1,000 non-viable eggs were injected into the small intestinal lamina propria, and lymph was collected on ice for 18 hours. As lymph cells were already in suspension, no tissue digest was necessary to obtain single cells for flow cytometric analysis. After cell straining, fluorescent monoclonal antibodies against B220, CD19, CD4, CD8, MHCII and CD11c and a dead cell exclusion dye were added and cells were analysed by flow cytometry. From total live cells, B cells were selected as $B220^+$ $CD19^+$ cells. $B220^-$ $CD19^-$ cells were separated into $MHCII^+$ $CD11c^+$ DCs and CD4 and CD8 T cells (Fig. 5-2 A). We observed that B cells comprised around 50% of all lymph cells in steady state,

whereas 20% were CD4 and 10% CD8 T cells and DCs represented around 1%. The frequency of these lymph migrating immune cells were compared between uninjected, PBS injected, and egg injected animals. Percentages of CD8 T cells and DCs increased slightly after egg injection whereas percentages of B cells decreased, but no statistical difference was observed (Fig. 5-2 B). As the thoracic duct also collects lymph draining from visceral tissues and lymph nodes other than the intestine, we could not determine what proportion of T and B cells derived from the intestine. However, we were confident that all DCs originated from intestinal tissues, as the MLNs were the only lymph nodes that had been removed in these animals. Thus, equal proportions of intestinal DCs migrated to the MLNs in steady state and during PBS or egg injection and could be collected in lymph of MLNx cannulated mice. We also recorded the total cell number for each sample and were able to calculate the total number of cells for each lymph population. In total around 5-8 million cells were collected per animal. However, cell numbers were somewhat variable, and were likely dependent on the overall quality of the surgical cannulation procedure. B cells accounted for 2-4 million cells and CD4 T cells were present at 1-2 million and CD8 T cells at 300,000-1 million cells. Around 60,000-200,000 lymph DCs were collected from uninjected, PBS injected or egg injected mice (Fig. 5-2 C). Again no difference in cell numbers could be observed between the different groups of mice. Thus we could demonstrate that thoracic duct cannulation is a powerful procedure that allows the direct assessment of lymph migratory immune cells. We furthermore established that intestinal egg injections did not have a significant effect on the migration of B cells, T cells and total DCs in lymph compared to steady state or PBS injected control groups.

5.1.2 In lymph intestinal egg antigens are transported by dendritic cells and B cells

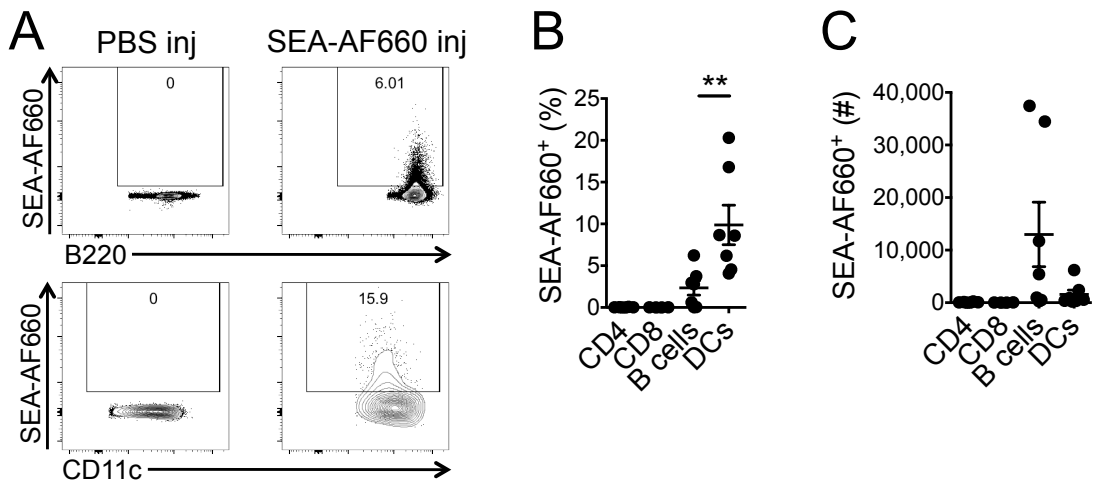

Figure 5-3. Lymph migrating DCs and B cells transport SEA from the small intestine to the MLNs

Uptake of SEA was measured by conjugating fluorescent AF660 to SEA. AF660 signal in $B220^+$ B cells and $CD11c^+$ $MHCII^{hi}$ DCs is shown in lymph collected from PBS or SEA-AF660 injected mice 18 hours after injection (A). Percentage (B) and total number (C) of lymph migrating CD4 and CD8 T cells, B cells and DCs that carry labelled antigen after SEA-AF660 injection in the small intestine. Data represent three independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied between B cells and DCs for statistical analysis (**P ≤ 0.01).

We had observed that large numbers of immune cells could be identified in lymph from uninjected mice and that intestinal egg injections did not induce any significant changes in cell migration. This suggested that steady state migratory cells were sufficient to transport parasite antigen to the draining lymph nodes where they induced Th2 responses. In order to visualize lymph migratory cells that were responsible for transporting egg antigen to the MLNs, we fluorescently labelled soluble egg antigen with Alexa Fluor 660. We injected 20 µg of AF660-SEA into the lamina propria of cannulated MLNx mice. This amount was equivalent to the amount of SEA used in priming experiments and thus represented an immunologically relevant quantity (see chapter 3). Lymph was collected for 18 hours after injection and CD4 and CD8 T cells, $B220^+$ B cells and total $CD11c^+$ $MHCII^{hi}$ DCs stained for flow cytometry and analysed. Uptake of SEA by these cells was quantified by the level of AF660 fluorescence and SEA-$AF660^+$ cells were selected by gating on $AF660^+$ cells that were present in SEA-AF660 injected mice but not in PBS injected controls (Fig. 5-3 A). Cells from PBS injected

controls showed no background fluorescence, whereas AF660 fluorescence was clearly detectable in lymph cells from SEA-AF660 injected mice. CD4 and CD8 T cells did not acquire any detectable fluorescence, whereas around 3% of B cells and 10% of total DCs were AF660$^+$ in lymph (Fig. 5-3 B). As B cells are far more abundant than DCs, the total number of AF66-carrying B cells reached up to 40,000 whereas SEA$^+$ DCs accounted for 1,000-4,000 cells (Fig. 5-3 C).

By cannulating AF660-SEA injected mice we were able to directly monitor lymph migrating cells and observed that both DCs and B cells transported intestinally injected SEA in lymph.

5.1.3 Distinct subsets of intestinal dendritic cells migrate in increased frequency after injection of eggs in the small intestine or colon and transport egg antigens to the respective draining lymph nodes

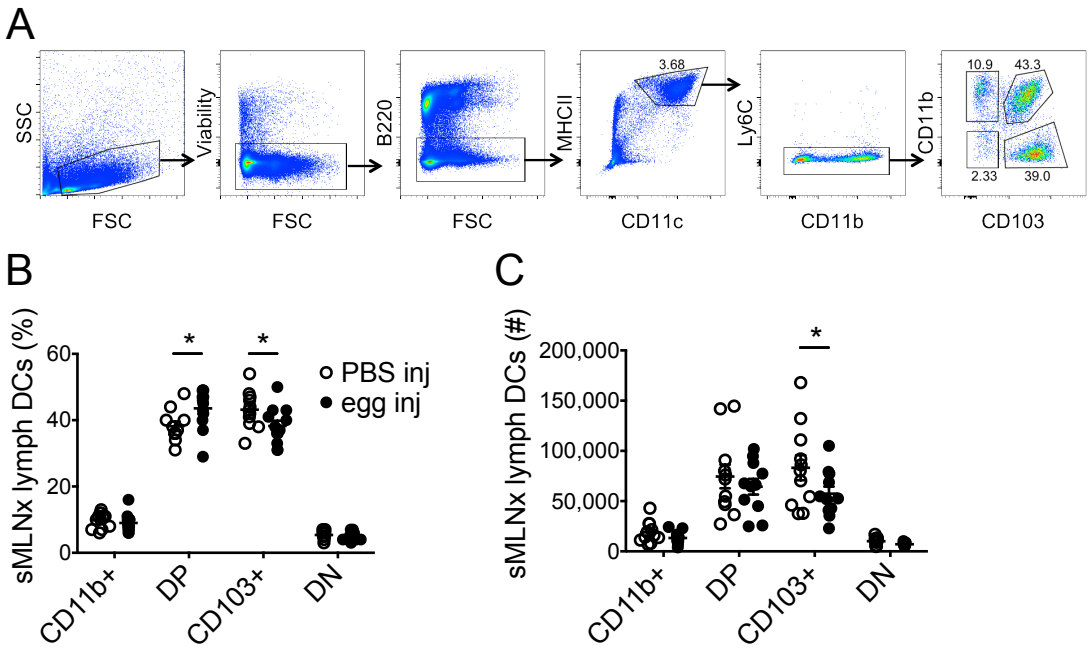

Figure 5-4. Egg injections into the small intestine affect the frequency of migrating lymph DC subsets

Lymph from sMLNx animals was stained for flow cytometry and CD11b and CD103 expressing DC subsets were identified using the gating strategy shown in (A). Frequency (B) and total number (C) of sMLNx lymph DC subsets was compared between PBS and egg-injected animals. Data represent at least three independent experiments (mean ± SEM) with each point representing one animal. Student's *t* tests were applied between DC subsets from PBS and egg injected animals for statistical analysis (*P ≤ 0.05).

We had observed in previous experiments using the IRF4$^{f/f}$ CD11c-cre mouse model that CD11c^{+} cells, which excludes B cells, were necessary for the induction of Th2 responses against *S. mansoni* eggs. Furthermore, we tested the functions of antigen-carrying B cells and DCs in additional experiments that are described in chapter 6 and found that only DCs were able to induce Th2 responses. We therefore concentrated on the effects of egg injection and antigen transport on DCs and their subsets, which are described in the experiments that follow. It has been reported that DC activation, for example by TLR agonists, induced the migration of all or certain DC subsets and assessed whether egg antigens induced a similar effect. We had observed that the injection of eggs did not cause changes in the migration pattern of total DCs in lymph when compared to uninjected and PBS injected animals. However, changes in certain DC subsets could have been masked when analysing total DCs.

In agreement with the literature, we had observed that different DC subset compositions are found in the small intestine and colon. In the small intestine DP DCs are the most abundant subset, whereas in the colon CD11b^{+} DCs are the majority subset. To separately assess the effects of egg antigen on these tissue-specific DC subsets, we performed thoracic duct cannulation experiments using mice from which only the sMLNs or cMLNs had been previously removed, allowing the collection of small intestinal or colonic derived DCs respectively. To determine if changes in small intestinal migrating DCs occurred after egg injection, sMLNx mice were cannulated and 20 µl PBS or 1,000 eggs were injected into the small intestine. Lymph was collected for 18 hours on ice and single cell suspensions stained with fluorescent monoclonal antibodies against B220, MHCII, CD11c, Ly6C, CD11b and CD103. DCs were identified as live B220^{-} MHCII^{hi} CD11c^{+} Ly6C^{-} single cells and represented 1-2% of all lymph cells. Subsets were distinguished by their expression of CD11b and CD103. All four subsets could be identified in lymph draining the small intestine. Hereby DP and CD103^{+} DCs were the most numerous subsets, each representing around 40% of sMLNx lymph DCs (Fig. 5-4 A). Around 10% of DCs were CD11b single positive and 2% were CD11b^{-} CD103^{-}. We compared lymph from PBS and egg injected animals and observed a significant increase in DP DCs after egg injection. Reciprocally, the percentage of CD103^{+} DCs decreased (Fig. 5-4 B), which suggested that the migration of DP DCs increased after egg injection into the small intestine. To better understand these changes we compared total cell numbers. Around 10,000 cells from each

cannulated animal were CD11b$^+$ DCs; 50,000-60,000 cells were DP or CD103$^+$ DCs and 1,000-2,000 DCs stained negative for both CD11b and CD103. However, the collected volume of lymph was quite variable between animals affecting the total number of cells. Thus, differences in the total number of DP DCs in lymph increased in egg injected mice but were no longer significant. However, the decrease in CD103$^+$ DCs was again observed to a significant degree (Fig. 5-4 C). Taken together these data indicate that upon egg injection in the small intestine DP DCs could be found at an increased frequency in lymph, suggesting that they might selectively respond to egg antigen leading to their enhanced migration.

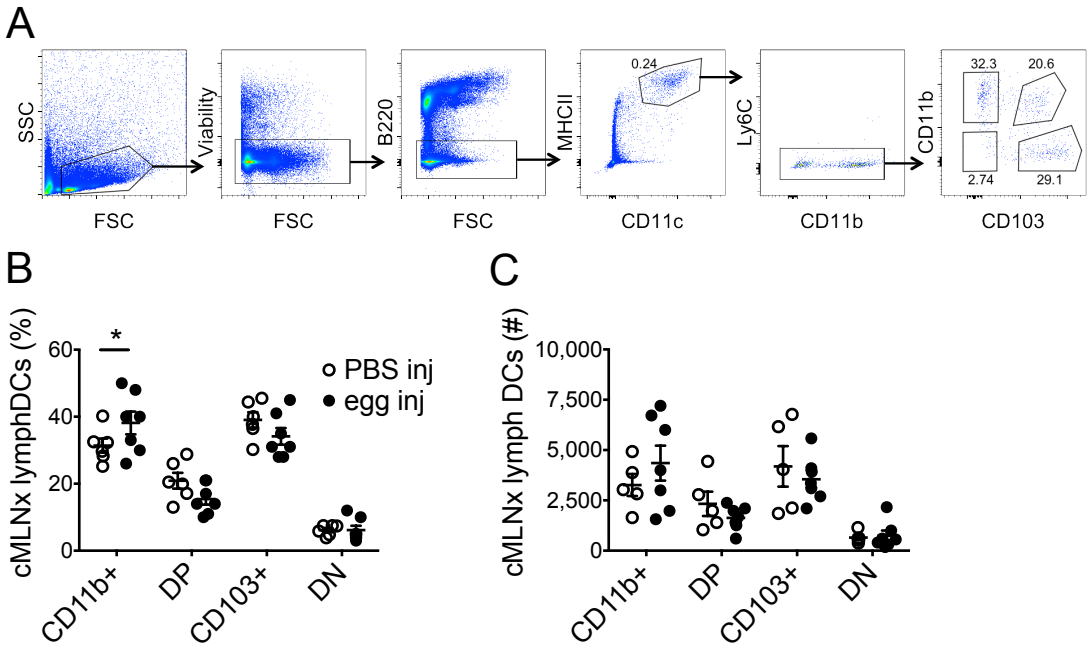

Figure 5-5. Increased frequencies of CD11b$^+$ cMLNx lymph DCs are observed after egg injections into the colon

Lymph from cMLNx animals was stained for flow cytometry and DC subsets identified by their expression of CD11b and CD103 using the presented gating strategy (A). Frequency (B) and total number (C) of cMLNx lymph DC subsets was compared between PBS and egg-injected animals. Data represent three independent experiments (mean ± SEM) with each point representing one animal. Student's *t* tests were applied between DC subsets from PBS and egg injected animals for statistical analysis (*P ≤ 0.05).

To assess whether the migration of specific DC subsets that migrate from the colon was also affected by egg injection, 20 µl of PBS or 1,000 *S. mansoni* eggs were injected into the colonic lamina propria of cMLNx mice. Thoracic duct lymph was collected from cannulated animals for 18 hours and stained for flow cytometry. DCs were again identified as live B220$^-$ MHCII^{hi} CD11c$^+$ Ly6C$^-$ single

cells. As already observed in the colonic lamina propria and the cMLNs, DCs were present at a much lower frequency compared to their small intestinal counterparts. The total number of cells collected from cMLNx animals was comparable to those from sMLNx mice, however the percentage of DCs was 10-fold lower. All four subsets of DCs could be identified in lymph and their composition was comparable to those found in the colonic lamina propria or the cMLNs. $CD11b^+$ and $CD103^+$ subsets represented the majority of DCs with each comprising 30-40% of cMLNx lymph DCs, whereas DP DCs comprised 20% and DN DCs 3% (Fig. 5-5 A). In lymph collected from egg injected mice the percentage of $CD11b^+$ DCs increased from 30% to 40% compared to PBS injected controls, while DP and $CD103^+$ DCs became less frequent (Fig. 5-5 B). This increase was also observed in total cell numbers, but was not statistically significant, as variable volumes of lymph collected from each animal resulted in a high variability between samples. The total number of DCs hereby ranged from 500 DN DCs to 7,000 $CD11b^+$ cMLNx lymph DCs (Fig. 5-5 C), which was much lower compared to sMLNx lymph DC subsets, where up to 150,000 DP DCs could be collected from a single animal. Subset specific changes in lymph DCs were however observed upon egg injection in both sMLNx and cMLNx mice, suggesting a subset specific effect on DC migration. Surprisingly, DP DCs were increased in small intestinal draining lymph whereas $CD11b^+$ DCs were affected after colonic egg injection. We therefore hypothesized that small intestinal DCs and colonic DCs respond differently to *S. mansoni* egg injection.

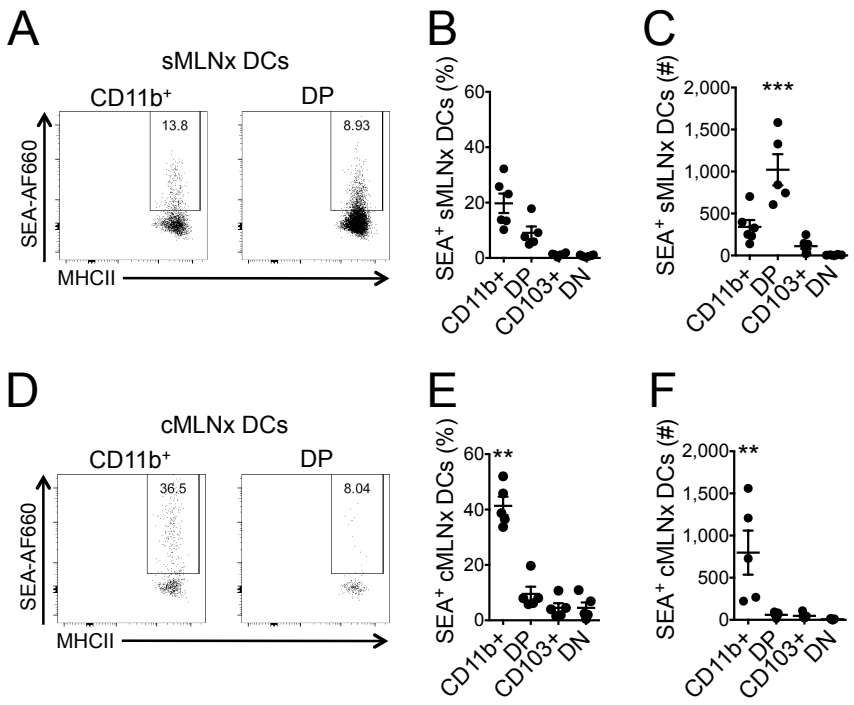

Figure 5-6. DP DCs in the small intestine and CD11b$^+$ DCs in the colon are the predominant subsets that carry *S. mansoni* egg antigen in lymph

Uptake of SEA-AF660 injected in the small intestine was assessed in lymph DC subsets from sMLNx mice (A). Frequency (B) and total number (C) of SEA$^+$ sMLNx DC subsets are shown. Conversely, SEA-AF660 was injected in the colonic lamina propria of cMLNx mice and lymph collected for 18 hours. Uptake of antigen was measured by AF660 fluorescence in lymph DC subsets (D). Frequency (E) and total number (F) of SEA$^+$ cMLNx DC subsets are shown. Data represent three independent experiments (mean ± SEM) with each point representing one animal. Kruskal-Wallis tests were applied for statistical analysis (**P ≤ 0.01; ***P ≤ 0.001).

As we had observed that lymph DCs transported parasite antigen from the lamina propria to the MLNs and that specific DC subsets selectively responded to egg injection in the small intestine and colon, we assessed whether egg antigen was also selectively transported by specific DC subsets. To determine whether antigen was transported by specific DC subsets from the small intestine, SEA-AF660 was injected into the small intestinal lamina propria of sMLNx mice. Lymph was collected from cannulated mice for 18 hours and cells stained for flow cytometry. DC subsets were identified by their expression of CD11b and CD103 and uptake of antigen measured by AF660 fluorescence (Fig. 5-6 A). We observed that around 20% of CD11b$^+$ and 9% of DP DCs in lymph draining the small intestine were SEA-AF660$^+$, whereas CD103$^+$ and DN sMLNx lymph DCs did not carry AF660 (Fig. 5-6 B). Although a higher frequency of CD11b$^+$ DCs carried AF660, SEA-AF660$^+$ DP DCs were more numerous when we compared total numbers, as DP DCs are the most abundant subset in small intestinal lymph (Fig. 5-6 C).

Taken together with our previous findings, which demonstrated that DP DCs were more frequent in lymph after small intestinal egg injection, we hypothesized that DP DCs from the small intestine were specialized to respond to *S. mansoni* egg antigen and represented the main subset involved in inducing Th2 responses in the small intestine.

To determine which DC subset was involved in carrying antigen from the colonic lamina propria, 20 µg SEA-AF660 was injected into the colons of cMLNx mice. Mice were cannulated and lymph collected for 18 hours, after which cells were stained with fluorescent monoclonal antibodies and analysed by flow cytometry. The uptake of antigen was assessed by AF660 fluorescence in all four DC subsets and we observed that $CD11b^+$ DCs were the most efficient DC subset to take up labelled-SEA (Fig. 5-6 D). In fact, 40% of cMLNx lymph $CD11b^+$ DCs were SEA-$AF660^+$, whereas only 8% of DP DCs and 4% of $CD103^+$ and DN DCs carried SEA-AF660 in lymph after colonic injection (Fig. 5-6 E). When comparing total numbers of labelled DCs, $CD11b^+$ DCs again carried the majority of antigen in cMLNx lymph, whereas only very few cells from the other subsets were SEA-$AF660^+$ (Fig. 5-6 F). Together with our observation that the frequency of $CD11b^+$ DCs was increased after colonic egg injection, we suggest that $CD11b^+$ DCs in the colon specifically respond to egg antigen. As DP DCs in the small intestine and $CD11b^+$ DCs in the colon selectively respond to and transport *S. mansoni* egg antigen in lymph we hypothesize that they represent tissue-specific subsets specialized to induce Th2 responses in the respective draining lymph nodes.

5.1.4 Egg antigens only have limited effects on the expression of costimulatory markers by dendritic cells

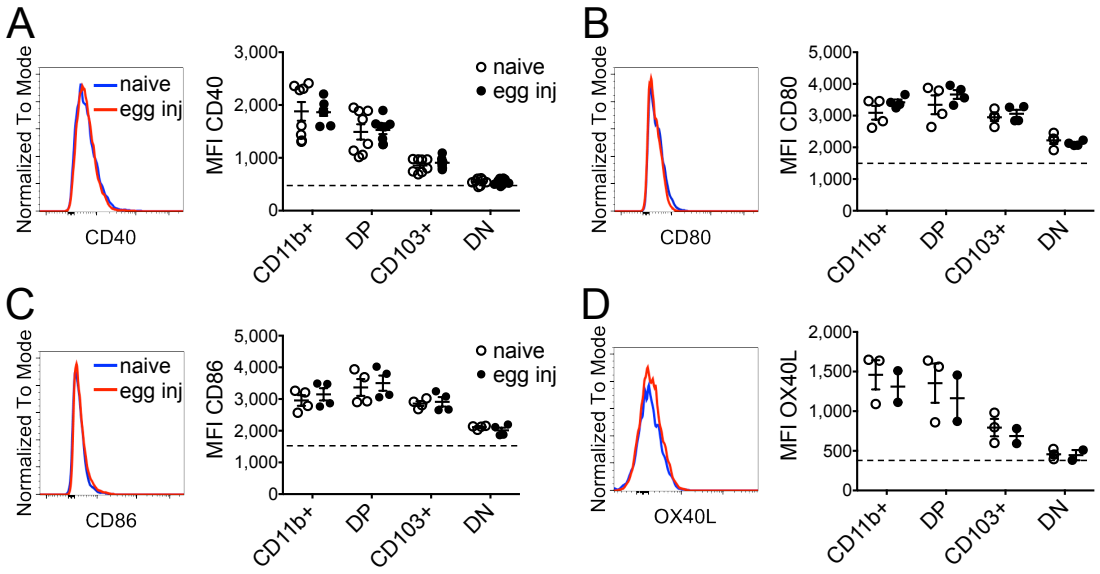

Figure 5-7. Egg injection does not induce the expression of CD40, CD80, CD86 or OX40L on lamina propria DCs

The expression of CD40 (A), CD80 (B), CD86 (C) and OX40L (D) was assessed on small intestinal LP DC subsets from naive and egg injected animals. Median fluorescent intensity of isotype controls is represented as a dotted line. Data represent at least two independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied between naive and egg injected groups for statistical analysis.

We have shown that egg antigen is taken up by specialized DC subsets in the small intestine and colon and transported to individual draining lymph nodes. There they interact with naive T cells and prime antigen specific immune responses. It has been reported that the uptake of antigen stimulates DCs and leads to the expression of costimulatory molecules that are required to efficiently communicate with naive T cells (Constant and Bottomly, 1997). We therefore addressed whether exposure to egg antigen also led to the upregulation of costimulatory molecules on DCs. We focused on CD40, CD80 and CD86, which represent costimulatory molecules that are upregulated during antigen-driven TLR activation, and OX40L which had been identified as a costimulatory molecule involved in Th2 priming (Jenkins et al., 2007). *S. mansoni* eggs were injected into the small intestinal lamina propria of C57BL/6 mice and the injected tissue was harvested after 24 hours. Tissues were digested with Collagenase VIII and cells stained for flow cytometry. The expression of costimulatory markers by DCs was assessed by measuring the median fluorescent intensity (MFI) for each antibody

compared to its fluorescent isotype control. Hereby a shift in fluorescent intensity signified increased expression. We observed that small intestinal DCs expressed low levels of CD40. Hereby, the $CD11b^+$ and DP DC subsets expressed higher amounts of CD40 compared to $CD103^+$ DCs but no increase in CD40 expression was observed in egg injected tissues (Fig. 5-7 A). The expression of CD80 and CD86 by small intestinal DCs was also low. Their MFI was around 3,000 for $CD11b^+$, DP and $CD103^+$ DCs and lower for DN DCs. As observed for CD40, egg injection did not have an effect on the expression of CD80 or CD86 by any of the DC subsets (Fig. 5-7 B&C). We hypothesized that the expression of these classical costimulatory markers, which are associated with proinflammatory conditions, were not induced by egg antigens. However, we assumed that egg antigens might induce the expression of OX40L, which had been associated with Th2 cell priming (Jenkins et al., 2007). We observed that OX40L was weakly expressed by small intestinal DCs and that $CD103^+$ DCs expressed lower levels than $CD11b^+$ and DP DCs and DN DCs did not express any OX40L. Furthermore, levels of OX40L did not increase in any of the small intestinal DC subsets upon egg injection (Fig. 5-7 D). Thus, we concluded that egg injection into the small intestine did not affect the overall expression of conventional or Th2 inducing costimulatory molecules on lamina propria DCs 24 hours after injection. However, we were unsure if the injection of eggs into the small intestine represented a suitable model to study costimulatory marker upregulation, as the distribution of antigen and cell migration away from the lamina propria could not be controlled.

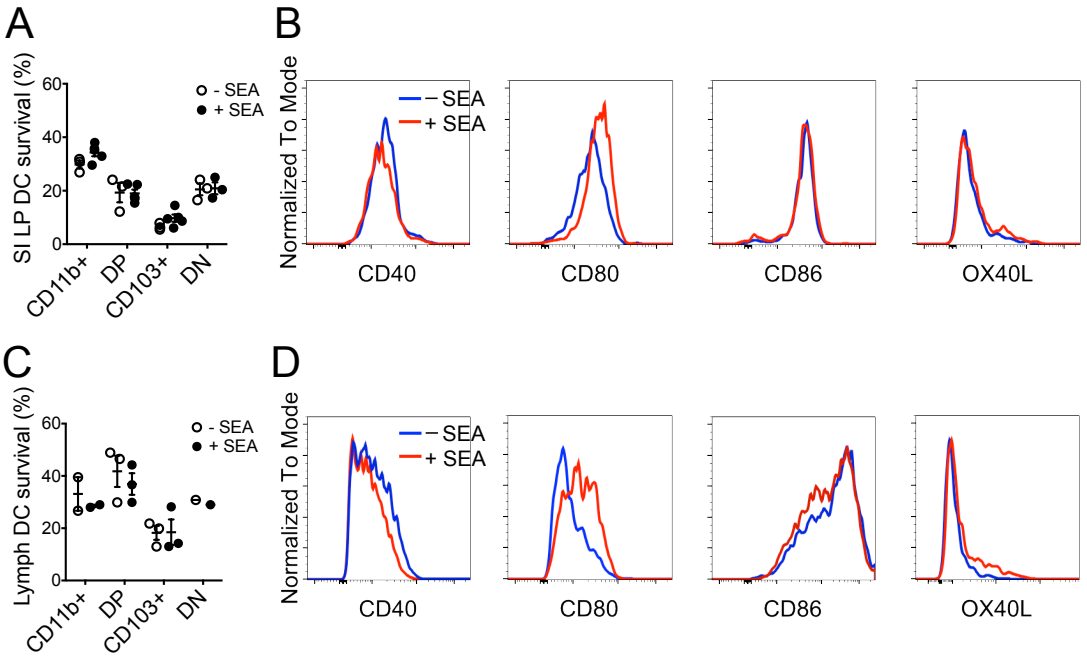

Figure 5-8. *In vitro* incubation with SEA only has limited effects on the expression of costimulatory markers of FACS sorted LP and lymph DCs

Survival of FACS sorted SI LP DC subsets after *in vitro* incubation with or without SEA (+/- SEA) for 18 hours (A). Representative expression of the costimulatory markers CD40, CD80, CD86 and OX40L is shown on SI LP DCs cultured with or without SEA for 18 hours (B). Viability of FACS sorted lymph DC subsets after *in vitro* incubation with or without SEA (+/- SEA) for 18 hours (C). Representative MFI histograms of lymph DCs incubated with or without SEA for 18 hours are shown for CD40, CD80, CD86 and OX40L (D). Data represent at least two independent experiments (mean ± SEM) with each point representing cells from an individual animal.

We had observed that the expression of the costimulatory markers CD40, CD80, CD86 and OX40L by lamina propria DCs was not affected by intestinal egg injection. However, egg injections might not represent a suitable model to detect changes in costimulatory marker expression, as not all DCs might be exposed to the injected antigen. Indeed, we have shown that only 9% of total DCs take up fluorescent SEA in the small intestine. Thus, potential responses could be masked. Furthermore, DCs constantly migrate from the lamina propria to the draining lymph nodes and might not be present in the tissue at the time of harvest. We therefore incubated FACS-sorted DCs with SEA *in vitro* to establish a controlled environment and ensure antigen availability to all cells. Small intestines of C57BL/6 mice were harvested and digested with Collagenase VIII. Cells were stained with fluorescent monoclonal antibodies against B220, MHCII, CD11c, CD64, Ly6C, CD103 and CD11b and total DCs purified by FACS sorting. DCs were incubated with or without 15 µg/ml SEA for 18 hours at 37° C. Cells were then stained for the costimulatory markers CD40, CD80, CD86 and OX40L and

7AAD staining solution was added to assess viability. We observed that after *in vitro* incubation the viability of small intestinal DC subsets was greatly affected. When gating on the individual DC subsets and assessing their viability we observed that 35% of $CD11b^+$ DCs were live, whereas 20% of DP and DN DCs and only 10% of $CD103^+$ DCs were viable after the 18-hour incubation. No difference in survival was observed in SEA-containing cultures, suggesting that cell-intrinsic characteristics of the different subsets rather than the presence of antigen affected their survival *in vitro* (Fig. 5-8 A). The expression of costimulatory markers was assessed by comparing the MFIs between cells that were incubated with or without SEA. We observed that the expression of CD40 on total small intestinal DCs slightly decreased with SEA incubation whereas CD80 expression increased. However, these changes in MFI were not statistically significant and the expression of CD86 and OX40L by small intestinal DCs was unchanged after SEA incubation (Fig. 5-8 B). Supporting our previous observations, these data indicated that *in vitro* incubation with *S. mansoni* egg antigen only had minor effects on the expression of costimulatory markers by small intestinal DCs. However, *in vitro* incubation also severely affected cell viability, which could negatively influence cell stimulation and we suspected that the enzymatic digestion during DC isolation from the small intestine had a negative effect on cell survival.

As DCs collected from the lymph do not require enzymatic digestion we tested whether incubation with SEA would increase their expression of costimulatory markers. Contrary to small intestinal DCs, lymph DCs do not represent immature peripheral DCs, but are of a semi-mature state and express higher levels of MHCII, costimulatory markers and CCR7. However, contrary to fully mature MLN DCs, they still respond to TLR ligands and can acquire the ability to drive T cell differentiation, supporting our approach to incubate them with SEA *in vitro* (Cerovic et al., 2013). Steady state lymph was collected from MLNx C57BL/6 mice and stained for flow cytometry. Total lymph DCs were sort purified and incubated with or without 15 µg/ml SEA for 18 hours. After incubation cells were stained for the costimulatory markers CD40, CD80, CD86 and OX40L and a viability dye was added. We observed that lymph DCs had a higher viability after the 18-hour incubation than their small intestinal LP DC counterparts, indicating that the enzymatic digestion of LP DCs negatively affected their survival. 35% of $CD11b^+$ lymph DCs, 40% of DP DCs, 20% of $CD103^+$ DC and 30% of DN DCs were viable after 18 hours and no statistical difference in cell survival was detected between

DCs incubated with or without SEA (Fig. 5-8 C). Compared to small intestinal LP DCs the expression of CD86 was higher in lymph DCs, indicating their semi-mature state. Incubation with SEA decreased the expression of CD40 and CD86, whereas the MFIs of CD80 and OX40L were increased (Fig. 5-8 D). However, due to insufficient experimental replicates statistical analysis could not be performed, but MFI differences between lymph DCs incubated with or without SEA were greater compared to their small intestinal counterparts. Thus, lymph DCs expressed lower levels of CD40 and CD86, while expressing increased amounts of CD80 and OX40L after *in vitro* incubation with *S. mansoni* egg antigen.

5.1.5 CD301b$^+$ CD11b-expressing dendritic cells preferentially carry egg antigens to the draining lymph nodes

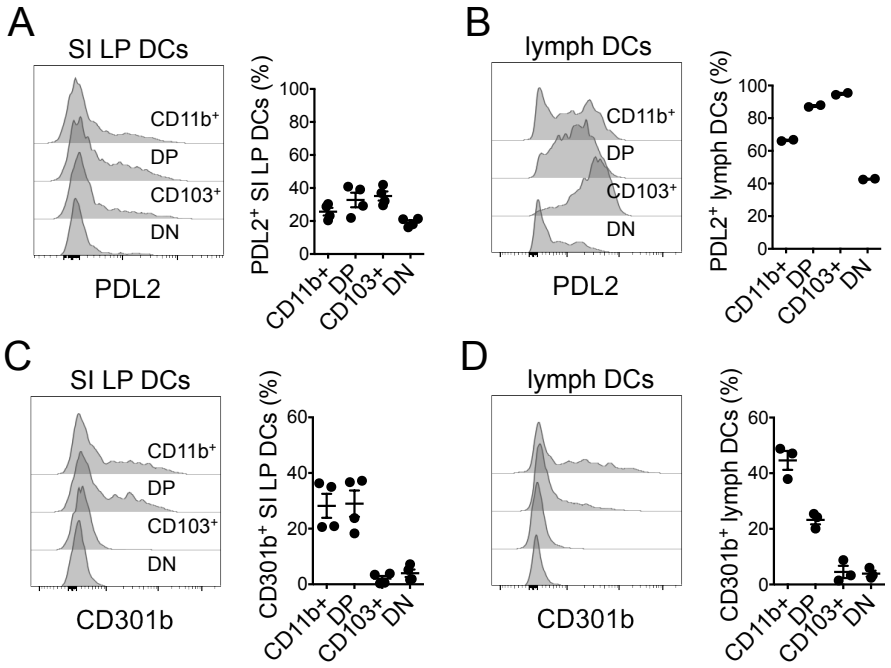

Figure 5-9. CD301b but not PDL2 expression matches the subset specific DC profile for intestinal Th2 induction

Representative MFI histograms and percentage of PDL2 expression by small intestinal (A) and steady state sMLNx lymph (B) DC subsets. CD301b expression by small intestinal (C) and sMLNx lymph (D) DC subsets are shown in representative MFI histograms and the percentage of CD301b expression. Data represent two independent experiments (mean ± SEM) with each point representing one sample.

We had observed that the incubation with egg antigen affected the expression of costimulatory molecules on DCs, and as such induced the expression of CD80 and OX40L, whereas decreasing the expression of CD40 and CD86. We continued to characterize intestinal DCs by examining the expression of surface molecules that had previously been associated with Th2 priming. It had been reported that PDL2-expressing DCs were involved in the maintenance of Th2 responses in the skin both *in vitro* and *in vivo* (Gao et al., 2013). We therefore investigated if PDL2 was also expressed by intestinal DCs and could serve as a marker to identify Th2-inducing DC subsets. We harvested small intestines from wild-type C57BL/6 mice and digested them with Collagenase VIII. Cells were stained for flow cytometry and PDL2 expression analysed on DC subsets. We observed that around 30% of CD11b$^+$, DP and CD103$^+$ DCs expressed PDL2 in

the small intestine, whereas only 15% of DN DCs were PDL2$^+$ (Fig. 5-9 A). In previous experiments we had observed that CD11b$^+$ and DP DCs carried small intestinal egg antigen in lymph and therefore tested if these lymph DC subsets also expressed PDL2. We cannulated MLNx C57BL/6 mice and collected lymph for 18 hours. Cells for stained with fluorescent monoclonal antibodies and analysed by flow cytometry. A high percentage of DCs expressed PDL2 in steady state lymph. In fact, 65% of CD11b$^+$ DCs, 85% of DP DCs, 95% of CD103$^+$ DCs and 40% of DN DCs were PDL2$^+$ (Fig. 5-9 B). The expression of PDL2 on CD103$^+$ and DN DCs however suggested that PDL2 was not a marker that could selectively identify Th2 priming DCs, as our experiments suggested that only CD11b$^+$ and DP DCs were involved in carrying egg antigen to the draining lymph nodes. Rather, the increased level of PDL2 expression in lymph DCs suggested that PDL2 was a DC maturation marker. It had indeed been published that PLD2 expression was upregulated upon *in vitro* stimulation of bone marrow derived DCs, suggesting its involvement in DC maturation (Gao et al., 2013).

Another marker that had been associated with Th2 induction on DCs was CD301b. It had been shown that dermal CD301b$^+$ DCs drove Th2 responses in the skin and that CD301b expression was under the control of IRF4 (Kumamoto et al., 2013). From our own experiments we knew that both CD11b$^+$ and DP DCs expressed IRF4 and were important for the induction of intestinal Th2 responses. We therefore examined if these DC subsets also expressed CD301b. We harvested the small intestines of C57BL/6 mice and digested them with Collagenase VIII. Stained cells were analysed by flow cytometry and CD301b expression assessed on DC subsets. We observed that 30% of CD11b$^+$ and DP DCs in the small intestine expressed CD301b, whereas CD103$^+$ and DN DCs did not express CD301b, confirming published findings that CD301b was only expressed by IRF4$^+$ CD11b$^+$ cells (Fig. 5-9 C). To investigate if CD301b expression was altered in migrating DCs we assessed its expression profile on lymph DC subsets. Steady state lymph was collected from cannulated MLNx mice for 18 hours and stained for flow cytometry. 40% of lymph CD11b$^+$ DCs and 20% of DP DCs were CD301b$^+$, whereas CD103$^+$ and DN DC subsets did not express CD301b (Fig. 5-9 D). As the expression of CD301b did not markedly change between small intestinal and lymph DCs, it suggested that a committed subset of CD11b$^+$ and DP DCs expressed CD301b. Thus, CD301b was expressed on the same DC subsets that transported egg antigen to the MLNs, and could potentially help identify DCs

that were specialized for the uptake and presentation of parasite antigen and the induction of Th2 responses, as previously suggested in the skin (Kumamoto et al., 2013).

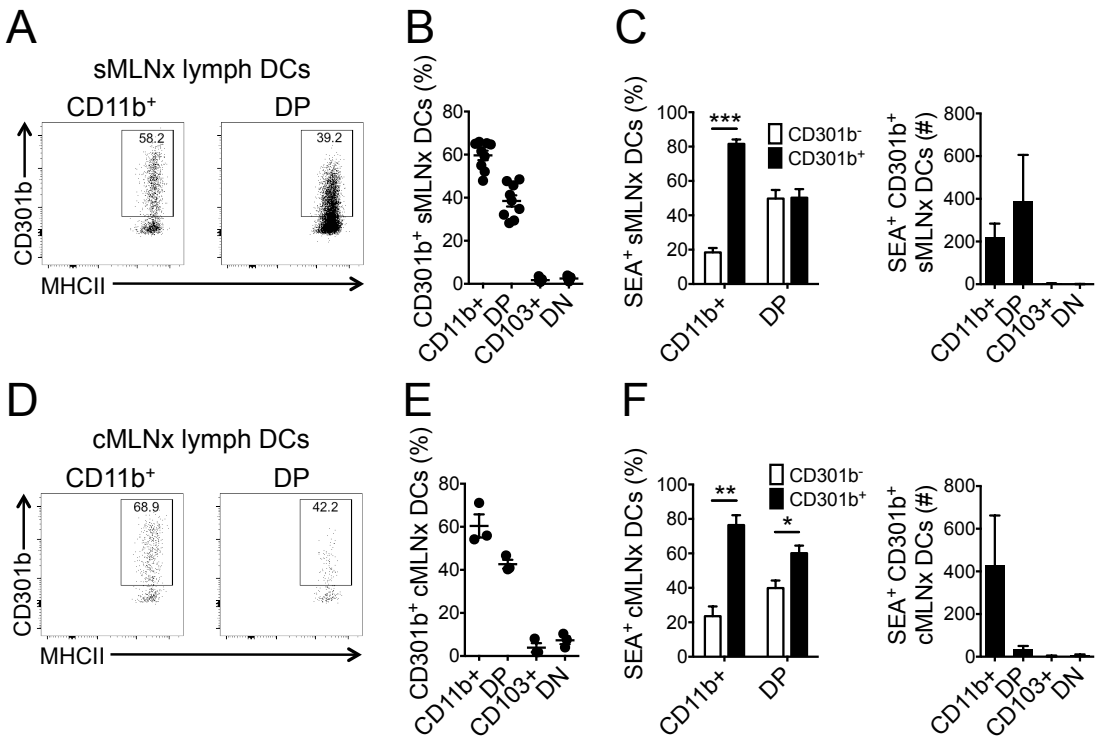

Figure 5-10. Subsets of CD11b$^+$ and DP DCs express CD301b in lymph and preferentially carry SEA

Expression of CD301b was assessed in sMLNx lymph DC subsets (A). Frequency of expression in each subset (B), uptake of SEA in CD301b$^-$ or CD301b$^+$ CD11b$^+$ and DP DCs and total number of SEA$^+$ CD301b-expressing cells of each DC subset (C) are shown. Equally, CD301b expression was also assessed in lymph DC subsets from cMLNx mice (D). Frequency of CD301b expression in each DC subset (E), uptake of SEA in CD301b$^-$ or CD301b$^+$ CD11b$^+$ and DP DCs and total number of SEA$^+$ CD301b-expressing cells of each DC subset (F) were evaluated. Data represent at least two independent experiments (mean ± SEM) with each point representing one animal (B&E) or with at least three animals per group (C&F). Mann-Whitney *U* tests between CD301b$^-$ and CD301b$^+$ groups (C&F) were applied for statistical analysis (*P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001).

It has been reported that CD301b$^+$ DCs are important to drive Th2 immunity in the skin (Kumamoto et al., 2013) and we had observed that that a committed subset of CD11b$^+$ and DP DCs expressed CD301b. These subsets were also responsible in transporting intestinally injected egg antigen to the MLNs, where they likely prime antigen specific Th2 responses. To investigate whether CD301b-expressing CD11b$^+$ and DP DCs were specifically involved in this process and would carry parasite antigen from the lamina propria to the draining lymph nodes we analysed

lymph DCs of SEA-AF660 injected mice. In previous experiments we had identified that antigen was carried by $CD11b^+$ and DP DCs in lymph. More precisely, DP DCs carried the majority of antigen injected in the small intestinal, whereas $CD11b^+$ DCs transported antigen from the colon. To address whether these organ specific differences could also be observed in the pattern of CD301b expression by DCs, we analysed small intestine and colon-draining lymph. sMLNx mice were cannulated and 20 µg SEA-AF660 injected in the small intestinal lamina propria. Lymph was collected for 18 hours and cells were stained for flow cytometry. When we assessed CD301b expression by DCs, we observed that it was expressed by 60% of $CD11b^+$ sMLNx lymph DCs and 40% of DP DCs (Fig. 5-10 A). $CD103^+$ and DN DCs did not express CD301b, which was in agreement with our previous findings (Fig. 5-10 B). To assess whether $CD301b^+$ DCs were specialized to take up egg antigen and prime Th2 responses we investigated whether they carried parasite antigen. The majority of SEA-AF660 staining was found in $CD301b^+$ DCs. 80% of SEA, that had been injected in the small intestine, was carried by CD301b-expressing $CD11b^+$ DCs and equal proportions of SEA were transported by $CD301b^-$ and $CD301b^+$ DP DCs. When we compared total numbers of SEA^+ $CD301b^+$ DC subsets we noticed that the majority of SEA was carried by sMLNx lymph $CD301b^+$ DP DCs and to a lower extend by $CD11b^+$ DCs, whereas $CD103^+$ and DN DCs did not carry egg antigen (Fig. 5-10 C). Our data therefore suggested that $CD301b^+$ DC subsets preferentially carried parasite antigen in sMLNx lymph and, in accordance with our previous findings, more DP DCs carried small intestinally injected antigen than $CD11b^+$ DCs.

We compared these findings to cMLNx mice that were cannulated and injected with 20 µg SEA-AF660 into the colonic lamina propria. In cMLNx lymph DCs, collected after 18 hours, we observed a similar CD301b expression profile as in sMLNx lymph. CD301b was expressed by 60% of $CD11b^+$ and 40% of DP DCs but not by $CD103^+$ and DN DCs (Fig. 5-10 D&E). Within the $CD11b^+$ DC subset, 80% of injected antigen was carried by CD301b-expressing cells and 60% of SEA was carried by CD301b cells within DP DCs, again demonstrating that the majority of SEA was carried by CD301b-expressing cells. In accordance to our previous findings and contrary to the small intestine, analysis of total numbers of SEA^+ $CD301b^+$ DCs revealed that the majority of SEA^+ cells in colon draining lymph were $CD11b^+$ DCs (Fig. 5-10 F). We had therefore established that CD301b was expressed by the majority of lymph $CD11b^+$ and DP DCs and that CD301b-

expressing DCs preferentially carried parasite antigen. Total numbers of antigen carrying DCs revealed tissue specific differences that were in accordance with our previous findings and showed that SEA^+ $CD301b^+$ DP DCs were more abundant in sMLNx lymph whereas CD301b-expressing $CD11b^+$ DCs carried nearly all SEA in cMLNx lymph. However, a significant proportion of $CD301b^-$ cells also carried SEA-AF660 in lymph suggesting that CD301b-expression by DCs is not sufficient to exclusively identify egg antigen presenting and Th2 priming DCs in our system.

5.2 Discussion

We had observed that the role of migrating DCs was essential for the induction of Th2 responses against intestinally injected *S. mansoni* eggs and that these responses were impaired in IRF4$^{f/f}$ CD11c-cre-positive mice, which exhibited reduced numbers of CD11b^{+} and DP DCs in the MLNs. To focus directly on the cells that migrated from the lamina propria to the MLNs and monitor their migrating behaviour we collected thoracic duct lymph from mesenteric lymphadenectomized mice. In these mice the removal of the MLNs ensures the migration of DCs to the thoracic duct, which is cannulated by the surgical insertion of a cannula (Cerovic et al., 2013). By selectively removing the sMLNs or cMLNs we could further collect only small intestine or colon-draining DCs in lymph (Houston et al., 2016). We observed that four main immune cell populations could be identified in lymph: B cells, CD4 and CD8 T cells, and DCs. B cells represented the majority of cells, followed by CD4 T cells and CD8 T cells, whereas DCs only represented 1-2% of all lymph cells (Fig. 5-2). Many of these B and T cells were likely recirculating throughout the lymphatic system and the thoracic duct and did not directly originate from the intestine. The collected DCs however were directly migrating from the intestinal lamina propria, as the MLNs were the only lymph nodes removed in these mice.

Thoracic duct cannulations have been used as a method to collect lymph from several animal species and similar observations have been made in lymphadenectomized mice and rats. It was demonstrated that mesenteric lymphadenectomy in rats was followed by the unification of the peripheral and central lymphatics, allowing the collection of intestine-derived peripheral lymph cells via the thoracic duct for several days. B and T cells comprised the majority of collected lymph cells, but nonlymphoid cells (NLC) that showed irregular and heterogeneous surface morphology, including long pseudopodia and veils, were also observed. These NLCs were present at very low frequencies in normal thoracic duct lymph at all times following cannulation and their expression of MHCII but lack of B or T cell markers suggested that they represent migrating DCs (Pugh et al., 1983). Similarly, thoracic duct cannulation of mesenteric lymphadenectomized mice allowed the collection of migrating DCs from the intestine, which represent 1-2% of all lymph cells and are absent in the lymph of non-lymphadenectomized mice (Cerovic et al., 2013).

Although steady-state migration of DCs occurs under homeostatic conditions it has also been reported that different TLR stimuli enhance the migration of DCs from the lamina propria to the draining lymph nodes. For example, oral administration of the TLR7/8 ligand resiquimod (R-848) induced a 20- to 30-fold increase in DC migration from the intestine within 10 hours, which was observed in the lymph of mesenteric lymphadenectomized rats and the mesenteric lymph nodes of mice. Hereby, R-848 induced the migration of nearly all lamina propria DCs resulting in cell accumulation in the MLNs (Yrlid et al., 2006). Similarly, intraluminal injection of *Bacillus subtilis* spores in MLNx rats induced a 5- to 10-fold increase in lymph migrating DCs, which selectively carried fluorescently labelled spores in lymph (Cerovic et al., 2009).

To test whether *S. mansoni* eggs affected the migration of immune cells, eggs were injected into the lamina propria of cannulated MLNx mice and lymph collected for 18 hours. We observed that the injection of PBS or eggs did not significantly affect the proportion or total number of migrating immune cells, suggesting that the injection in itself and the injection of eggs specifically did not impair or enhance cell migration in this setup (Fig. 5-3). To investigate the migration of DCs in more detail we distinguished between DCs originating from the small intestine or the colon. As DCs cannot migrate past the MLNs, the selective removal of the sMLNs ("sMLNx") allows the collection of small-intestine-derived DCs in thoracic duct lymph, whereas cMLN removal ("cMLNx") only enables colonic DCs to migrate to the thoracic duct (Houston et al., 2016). We observed that lymph draining the small intestine of egg-injected animals contained a higher frequency of DP DCs and a lower frequency of $CD103^+$ DCs compared to PBS injected controls. However, the volumes of lymph collected from each animal after cannulation of the thoracic duct were somewhat variable, as they are affected by surgical, physiological and micro-anatomical features of the individual cannulated animals. Therefore, changes in total numbers were less pronounced (Fig. 5-4). The finding that small intestinal DP DCs were found in increased frequency in the lymph of small intestinally egg-injected mice, suggested that this subset specifically responded to the injected antigen. This change in subset composition had likely been masked in previous experiments, where total DC frequencies and numbers were investigated, and demonstrated that *S. mansoni* egg antigen

stimulates DCs and specifically enhances the migration of the DP DC subset in the small intestine.

When we assessed the frequency and total number of DCs in colon draining lymph we observed that 10 times fewer DCs were present compared to sMLNx lymph. This observation was consistent with our data from the colonic lamina propria and the cMLNs which also contain fewer DCs. Furthermore, as previously reported in the colon and the cMLNs (Denning et al., 2011; Houston et al., 2016), $CD11b^+$ and $CD103^+$ DCs were the most abundant DC subsets in lymph draining the colon, in contrast to small intestinal lymph where DP and $CD103^+$ DCs are the most numerous subsets. Surprisingly, egg injection into the colon resulted in an increased frequency of migrating $CD11b^+$ DCs in the lymph of cMLNx mice and not in DP DCs, as previously observed in small intestinal draining lymph (Fig. 5-5). Therefore, egg injections had an effect on the migration of tissue specific DC subsets, increasing the percentage of small intestinal DP DCs and colonic $CD11b^+$ DCs in lymph of egg-injected animals.

To investigate whether this increase in DC subset migration corresponded with the uptake of injected antigen, fluorescently labelled SEA was injected in the lamina propria of sMLNx or cMLNx mice and lymph collected for 18 hours. Among DCs carrying labelled SEA from the small intestine, lymph DP DCs were the most numerous, whereas $CD11b^+$ DCs were the dominant subset that carried SEA injected in the colonic lamina propria (Fig. 5-6). Together with our previous observations that the same tissue specific subsets migrated in increased frequency after egg injection, we suggest that they are primarily involved in the uptake, transport and presentation of intestinal egg antigen to the MLNs. We therefore hypothesize that the priming of Th2 cells in the MLNs is likely to be carried out by distinct subsets of DCs; $CD11b^+CD103^+$ DP DCs in the small intestine and $CD11b^+CD103^-$ single positive DCs in the colon. If this hypothesis is confirmed, it would demonstrate that specific DC subsets are specialized for the induction of Th2 responses, a concept that has already been shown for other types of immune responses, and furthermore suggest a tissue specific specialization. This would raise the question why these DC subsets have differentially developed but serve the same function and bring new complexity to our understanding of the tissue-specific cues that influence DC differentiation and function in the intestine.

Once DCs reach the lymph nodes they present antigen to naive T cells and prime them against the antigen. However, for the proper differentiation into T helper cells naive T cells also require cues from costimulatory markers expressed by DCs. As such, conventional costimulatory molecules such as CD40, CD80 and CD86 are upregulated in response to proinflammatory TLR stimuli of bacterial, fungal or viral origin and induce the differentiation of Th1 and Th17 cells (Chen and Flies, 2013). The costimulatory molecule OX40L has recently been identified to play a role in Th2 immunity and its expression can be induced by TSLP or parasite products (Jenkins et al., 2007). We therefore assessed whether egg antigens also induced the expression of conventional costimulatory molecules or OX40L 24 hours after small intestinal egg injection but could not observe any changes in their expression profile on intestinal DC subsets (Fig. 5-7). However, our previous experiments had shown that only 9% of total DCs take up fluorescent SEA in the small intestine suggesting that not all DCs within the lamina propria are exposed to intestinally injected antigen. As subserosal egg injections do not cover the entire area of the lamina propria nor the entire length of the small intestine certain areas remained unaffected. Furthermore, lamina propria DCs constantly migrate to the MLNs and exposed DCs might no longer be present in the tissue at the time of harvest. To ensure antigen availability to all cells we incubated FACS-sorted DCs with SEA *in vitro*. We observed that *in vitro* culturing had a negative impact on cell survival and that small intestinal DC subsets were stronger affected than lymph DCs. We suspect that the enzymatic digestion of the small intestine had a negative effect on cell survival. However, cell survival was not affected by the addition of SEA, suggesting that cell-intrinsic characteristics of the different subsets affected their survival *in vitro*. We observed that the expression of CD40 on total small intestinal DCs slightly decreased with SEA incubation whereas CD80 expression increased. The expression of CD86 and OX40L by small intestinal DCs was unchanged.

When we assessed the expression of these costimulatory markers in lymph DCs, we observed a generally higher expression of CD86. As previously published, lymph DCs do not represent immature peripheral DCs but are of a semi-mature state and express higher levels of MHCII, costimulatory markers and CCR7. However, contrary to fully mature MLN DCs, they still respond to TLR ligands and can acquire the ability to drive T cell differentiation (Cerovic et al., 2013), suggesting that they could still respond to egg antigens *in vitro.* Similar to our

observations in small intestinal DCs, SEA incubation of lymph DCs decreased the expression of CD40 and increased the expression of CD80. In addition, levels of CD86 were reduced and the MFIs of OX40L were increased after SEA incubation (Fig. 5-8). However, due to insufficient experimental replicates statistical analysis could not be performed, but MFI differences between lymph DCs incubated with or without SEA were greater compared to their small intestinal counterparts. We therefore concluded that SEA only had minor effects on the expression of costimulatory markers by small intestinal and lymph DC subsets after *in vitro* incubation.

The observation that SEA does not increase the expression of conventional costimulatory markers has also been observed in the literature. In contrast to BMDCs incubated with *Propionebacterium acnes*, SEA-exposed BMDCs did not upregulate the costimulatory markers CD40, CD80 or CD86 (MacDonald et al., 2002). Similarly, human monocyte-derived DCs did not upregulate CD80, CD83 or CD86 after SEA incubation and TLR-induced expression with poly-I:C or LPS was downregulated by SEA (van Liempt et al., 2007). In contrast, several studies have shown that OX40L expression by DCs is upregulated by TSLP or parasite products and is an important signal that drives Th2 immunity. Thus, blocking of OX40L using monoclonal antibodies led to impaired Th2 differentiation by TSLP-activated human monocyte-derived DCs *in vitro* (Soumelis et al., 2002). Additionally, OX40L expression of human monocyte-derived DCs was increased after SEA or cholera toxin incubation (de Jong et al., 2002). Furthermore, murine SEA-pulsed OX40L$^{-/-}$ BMDCs could not induce antigen-specific Th2 responses when transferred into naive recipient animals, but responses were rescued by the injection of agonistic anti-OX40 antibodies, indicating that OX40L expression by DCs was important to drive appropriate Th2 responses (Jenkins et al., 2007). In our experiments we observed a minimal increase of OX40L expression after SEA incubation, but this increase was not as dramatic as some of the published responses. However, contrary to the culture systems used in the literature we assessed OX40L expression on tissue-derived DCs and not in *in vitro* generated cells, such as BMDCs or human monocyte-derived DCs. It needs to be taken into consideration that these *in vitro* generated cells represent a heterogeneous population of monocytes, DC-like and macrophage-like cells that may not accurately represent the behaviour of tissue-derived DCs (Helft et al., 2015). Although easy to generate, it is important to verify the observations in tissue-

derived DCs. We could therefore demonstrate that the incubation with SEA *in vitro* did not induce the expression of conventional costimulatory markers in intestinal tissue-derived or lymph DCs and only slightly increased the expression of OX40L.

Another DC marker that has recently been reported to be involved in Th2 immunity in the skin is PDL2. $PDL2^+$ BMDCs induced Th2 responses in effector/memory CD4 T cells *in vitro.* Moreover, PDL2 was highly expressed in skin LN DCs and $PDL2^+$ DCs induced the expression of IL-4 in naive and effector/memory CD4 T cells (Gao et al., 2013). We therefore assessed if PDL2 was also expressed in the intestine. We observed that around 30% of $CD11b^+$, DP and $CD103^+$ DCs and 15% of DN DCs expressed PDL2 in the small intestine and 65% of $CD11b^+$ DCs, 85% of DP DCs, 95% of $CD103^+$ DCs and 40% of DN DCs were $PDL2^+$ in lymph (Fig. 5-9). The expression of PDL2 on $CD103^+$ and DN DCs suggested that PDL2 was not a marker that could selectively identify Th2-priming DCs in our system, as our experiments suggested that only $CD11b^+$ and DP DCs were involved in carrying egg antigen to the draining lymph nodes. Rather, the increased level of PDL2 expression in lymph DCs suggested that PDL2 might be a DC maturation marker. It had indeed been published that PDL2 was upregulated upon *in vitro* stimulation of BMDCs and was highly expressed in skin draining lymph node DCs, suggesting its potential involvement in DC maturation (Gao et al., 2013).

Another marker that has been associated with Th2 induction in the skin is CD301b/Mgl2. It had been shown that $CD301b^+$ DCs carried OVA to the draining lymph nodes when the Th2-inducing adjuvant papain was administered, but not when PBS or the TLR-agonist CpG were injected. Furthermore, $CD301b^+$ cell deficient Mgl2-DTR mice did not respond to papain or alum immunization in the skin nor mounted Th2 responses against *N. brasiliensis*, whereas proinflammatory responses against CpG/OVA were unaffected (Kumamoto et al., 2013). When we assessed CD301b expression by intestinal DCs, we observed that a committed subset of $CD11b^+$ and DP DCs in the small intestine and lymph expressed CD301b (Fig. 5-9). CD301b was hereby expressed by $CD11b^+$ and DP DCs in both small intestinal and colonic draining lymph. Furthermore, CD301b-expressing DCs preferentially carried fluorescently-labelled egg antigen in lymph, similar to the reported uptake of OVA during papain administration. When we compared total numbers of antigen carrying DCs, we observed tissue specific differences that were in accordance with our previous findings. SEA^+ $CD301b^+$ DP DCs were

more numerous in sMLNx lymph whereas CD301b-expressing $CD11b^+$ DCs carried nearly all SEA in cMLNx lymph (Fig. 5-10). Thus, CD301b expression corresponded with the subset specific uptake of antigen we had previously observed. However, as the function of CD301b is currently unknown and a significant proportion of $CD301b^-$ cells also carried SEA-AF660 in lymph, CD301b-expression by DCs was not sufficient to identify Th2 priming DCs in our system.

Thus, our efforts to verify reported molecules that are important in the induction of Th2 responses have generated no clear result in our model of intestinal *S. mansoni* egg immunization. However, our experiments indicate that specific DC subsets are specialized to carry intestinal egg antigens to the draining lymph nodes. Interestingly, this is carried out by $CD11b^+CD103^+$ DP DCs in the small intestine and $CD11b^+CD103^-$ single positive DCs in the colon, which suggests a tissue specific specialization.

5.3 Conclusions

As previously published, we were able to identify B cells, T cells and DCs in the lymph of cannulated lymphadenectomized mice in steady state. The percentage and total number of these immune cells did not change upon intestinal egg injection suggesting that steady state migratory cells were sufficient to carry parasite antigen to the MLNs. Indeed, both migrating B cells and DCs carried fluorescent SEA injected into the small intestine in lymph. A closer look at the lymph migrating DC subsets revealed that DP and $CD103^+$ DCs were the most numerous DC subsets in small intestine-draining sMLNx lymph, whereas $CD11b^+$ and $CD103^+$ DCs were the most numerous populations migrating from the colon. However, the frequency of DCs in cMLNx mice was 10-fold lower compared to sMLNx lymph.

We observed that the frequency of the subset of DP DCs migrating from the small intestine increased after egg injection and that $CD11b^+$ DCs were more frequent after egg injection in the colon. Small intestinal DP DCs and colonic $CD11b^+$ DCs were also the most numerous subsets to carry fluorescent SEA in lymph, suggesting that the physiological differences between the small intestine and colon were reflected in distinct cellular responses upon *S. mansoni* egg injection. However, egg antigens did not induce the upregulation of conventional and Th2 associated costimulatory molecules on DCs after small intestinal egg injection. However, after *in vitro* incubation of FACS sorted small intestinal and lymph DCs with SEA, minor effects on the expression of CD40, CD80, CD86 and a slight upregulation of OX40L were observed. PDL2 expression of intestinal DC subsets, which has been associated with Th2 responses in the skin, was identified in all four subsets and increased in lymph, suggesting that it is associated with DC maturation rather than Th2 induction. CD301b expression, another Th2 associated molecule, however was only expressed on committed $CD11b^+$ and DP DCs in the small intestine and lymph, which correlated with our own findings that $CD11b^+$ and DP DCs were involved in intestinal Th2 responses. Indeed, $CD301b^+$ DCs in small intestinal and colonic draining lymph preferentially carried SEA and reflected tissue-specific differences in $CD11b^+$ and DP DC composition. Taken together, our data suggest that DP DCs from the small intestine and $CD11b^+$ DCs from the colon are specialized to carry intestinally injected egg antigen and are likely responsible for the induction of tissue specific Th2 responses in the respective MLNs.

Chapter 6: Different subsets of small intestinal and colonic dendritic cells drive Th2 responses upon transfer into recipient mice

6.1 Results

In our previous experiments we had observed that B cells and DCs transported fluorescently-labelled SEA from the intestine to the draining lymph nodes. We had furthermore shown that distinct subsets of DCs transported parasite antigen from the small intestine and colon. To identify if these cell populations were also directly involved in priming naive T cells in the MLNs we FACS purified these populations from egg injected donor mice and transferred them into the MLNs of wild type recipient animals. Importantly, the purified donor cells were precisely injected under the capsule of a single MLN, using techniques we have recently developed (Cerovic et al., 2015). This allowed the controlled delivery into the subcapsular sinus of the draining lymph node, their natural route of entry (Braun et al., 2011), enabling us to directly assess the priming capabilities of the transferred cells *in vivo*.

6.1.1 Dendritic cells but not B cells drive immune responses against *S. mansoni* egg antigens in the MLNs

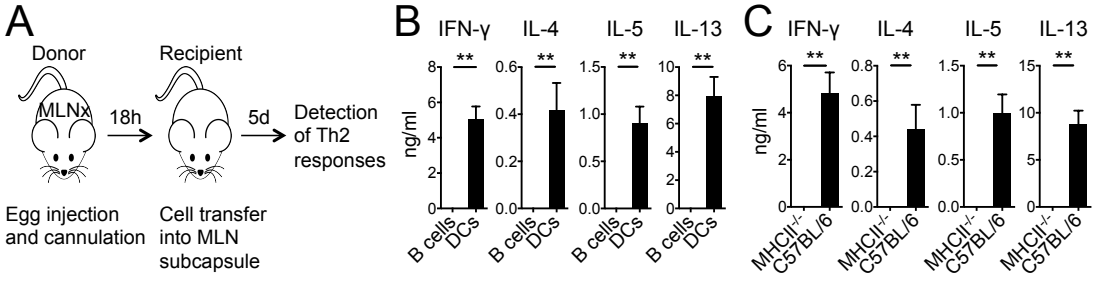

Figure 6-1. DCs but not B cells are sufficient to prime Th2 responses upon transfer into recipient mice

To assess whether lymph migrating immune cell populations could induce antigen specific immune responses once transferred into recipient mice, cells were collected from thoracic duct lymph of egg injected MLNx donor mice, FACS purified and transferred under the MLN capsule of recipient mice. 5 days after transfer immune responses were analysed by setting up 3 day *in vitro* MLN restimulation cultures. A schematic of the experiment is shown in A. Cytokines measured in restimulation cultures from transferred B cell and DC recipient mice (B). Immune responses induced by transferred MHCII$^{-/-}$ or C57BL/6 DCs from egg injected donors measured after restimulation of the recipient MLNs (B). Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (**P ≤ 0.01).

We had observed that both B cells and DCs, but not T cells, transported fluorescently labelled SEA in lymph, which had been injected into the subserosa of the small intestine of MLNx mice. Therefore, these cells were responsible for transporting the antigen from the intestine to the draining lymph nodes. To address if these cells were also sufficient to induce antigen specific immune responses within in the lymph node we transferred them into recipient mice. To collect these cells, eggs were injected into the intestines of donor MLNx mice and lymph was collected for 18 hours after cannulation. As we had observed that around 4% of B cells and 10% of DCs carried fluorescently labelled antigen, we equalized the amount of antigen-carrying cells by collecting twice as many B cells than DCs, and FACS purified 100,000 B cells and 50,000 total CD11c^{+} MHCII^{+} DCs from lymph. As these cells were collected from egg-injected donor mice, no exogenous antigen was provided but cells were directly resuspended in 5 µl PBS and injected under the MLN capsule of wild type recipient animals. The precise delivery into the MLN allowed us to investigate their physiological potential for priming T cells *in vivo*. We investigated if the transferred cells were sufficient to induce egg antigen specific

immune responses and 5 days after cell transfer the injected MLNs were harvested and cells were restimulated with SEA *in vitro*. After 3 days supernatants were collected and secreted cytokines measured by ELISA (Fig. 6-1 A). We detected the production of antigen specific cytokines in recipient mice after DC but not B cell transfer, suggesting that DCs but not B cells were sufficient to prime naive T cells in the recipient MLNs. IFN-γ as well as robust levels of IL-4, IL-5 and IL-13 were detected in restimulation cultures after DC transfer, indicating that egg antigen specific IFN-γ producing T cells as well as Th2 cells were primed by DCs but not B cells (Fig. 6-1 B). This observation corresponded to findings in the literature, which have shown in many experiments that antigen-presenting DCs are specialized to prime naive T cells and initiate immune responses, whereas antigen-presenting B cells communicate with effector/memory T helper cells to potentiate these responses. Furthermore, our own observations in $IRF4^{f/f}$ CD11c-cre mice indicated that CD11c-expressing cells, which include DCs but not B cells, are necessary to drive Th2 responses against *S. mansoni* egg antigens in the intestine.

As antigen presentation to CD4 T cells is mediated by MHCII molecules, we tested whether MHCII-deficient DCs could still induce immune responses upon transfer. It has been reported that uptake of antigen is not inhibited in $MHCII^{-/-}$ DCs, however antigen presentation to CD4 T cells does not occur (Villadangos et al., 2005). The transfer of $MHCII^{-/-}$ DCs from egg injected donors therefore allowed us to investigate if the transferred DCs directly primed naive CD4 T cells in the recipient animal or if they were just responsible for transporting the antigen to the draining lymph node. Again, eggs were subserosally injected into the small intestine of MLNx $MHCII^{-/-}$ or C57BL/6 mice and lymph collected via thoracic duct cannulations for 18 hours. Total $CD11c^{+}$ DCs were selected from live $B220^{-}$ $Ly6C^{-}$ $CD64^{-}$ cells and FACS purified. 50,000 DCs from either $MHCII^{-/-}$ or C57BL/6 donor mice were transferred under the MLN capsule of each recipient animal and 5 days after transfer the injected MLNs were harvested and restimulated for 3 days with SEA *in vitro.* IFN-γ, IL-4, IL-5 and IL-13 were measured in the supernatants of these restimulation cultures and gave an indication of the induced immune response. We observed that the transfer of $MHCII^{-/-}$ DCs did not induce any antigen specific immune responses in recipient mice, whereas the transfer of C57BL/6 DCs from egg injected donors induced robust IFN-γ and Th2 responses (Fig. 6-1 C). Therefore, our findings clearly indicate that DCs and not B cells were sufficient to

induce egg antigen specific immune responses after transfer into wild type recipient mice and were responsible for inducing Th2 responses against egg antigen. T cell priming was mediated directly by the transferred DCs through MHCII and did not occur when MHCII$^{-/-}$ DCs were transferred.

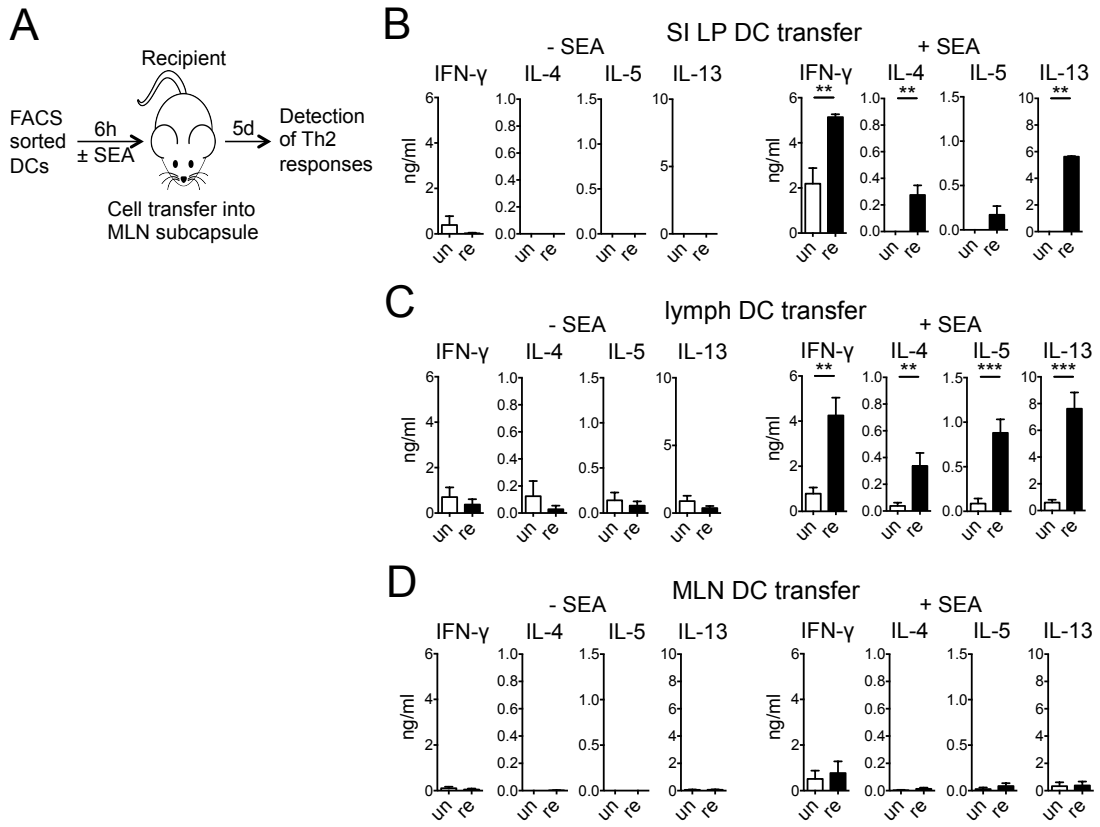

Figure 6-2. Small intestinal LP and lymph DCs, but not migratory MLN DCs, are able to present *in vitro* loaded SEA upon transfer

To assess if DCs could present *in vitro* loaded SEA upon transfer, DCs were purified by FACS sorting, incubated with or without SEA for 6 hours and transferred under the capsule of recipient mice. Induced immune responses were analysed using restimulation cultures from MLNs harvested 5 days after transfer. A schematic illustrating the experimental protocol is shown in A. Cytokines measured in recipient MLNs of small intestinal LP DCs loaded with SEA (+ SEA) or without SEA (- SEA) from unstimulated (un) or restimulated (re) wells (B). Immune responses induced by transferred lymph DCs (C) or MLN DCs (D) loaded with or without SEA *in vitro*. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (**P ≤ 0.01; ***P ≤ 0.001).

We had observed that lymph DCs from egg injected donor mice were sufficient to induce Th2 responses upon transfer into wild type recipient animals. In these experiments, antigen was acquired in the small intestinal lamina propria from subserosally injected eggs. To address whether *in vitro* antigen loaded DCs could

also be used to prime Th2 responses against SEA, we FACS sorted DCs from C57BL/6 mice, incubated them with our without SEA for 6 hours, and after thoroughly washing off any unbound antigen, transferred these cells under the MLN capsule of recipient mice. Antigen specific T cell responses were detected in the injected lymph nodes 5 days after DC transfer by *in vitro* restimulation and measurement of secreted cytokines (Fig. 6-2 A). We harvested total DCs from the small intestinal lamina propria, the lymph and the MLNs, which represent the tissues where migratory intestinal DCs are found (Cerovic et al., 2013). Small intestines of C57BL/6 mice were enzymatically digested with Collagenase VIII, cells stained with monoclonal fluorescent antibodies and total DCs purified by FACS. 50,000 total DCs were either incubated with or without 15 µg/ml SEA for 6 hours at 37°C *in vitro*. Cells were washed twice with PBS to remove any antigen that had not been taken up, and resuspended in 5 µl PBS. DCs were transferred under the MLN capsule of individual wild type recipient animals and the induction of antigen specific immune responses by the transferred cells measured 5 days after transfer using SEA restimulation cultures. We observed that small intestinal DCs that were not incubated with SEA did not induce any antigen specific immune responses in recipient mice. SEA loaded small intestinal DCs did on the other hand induce immune responses, measured by the secretion of IFN-γ, IL-4, IL-5 and IL-13 after restimulation. Low concentrations of IFN-γ (2 ng/ml) but no Th2 cytokines were detected in unstimulated wells, indicating that some cells secreted IFN-γ without being stimulated. However, much higher amounts of cytokine were detected upon restimulation and 5 ng/ml of IFN-γ, 0.2 ng/ml of IL-4 and IL-5 and 5 ng/ml of IL-13 were measured in these cultures (Fig. 6-2 B). Thus, small intestinal DCs that were loaded with SEA *in vitro* were sufficient to induce immune responses in recipient animals upon transfer. Similar to our previous experiments, these responses were antigen specific against SEA and required restimulation to be detected, indicating that antigen specific IFN-γ-producing cells and Th2 cells had been primed.

Small intestinal DCs represent immature peripheral DCs that are responsible for taking up antigen in the lamina propria, which likely enabled them to also sample antigen *in vitro*. Upon stimulation by intestinal antigen and homeostatic signals within the intestine they enter a semi-mature state and upregulate MHCII, costimulatory markers and CCR7 (Randolph et al., 2005). They then migrate through the lymphatics to the MLNs, to present the acquired antigen. To test

whether semi-mature lymph DCs were also able to take up and present antigen acquired *in vitro*, we cannulated the thoracic lymph duct of MLNx animals and collected the lymph for 18 hours. Cells were then stained with fluorescent antibodies and total DCs purified by FACS sorting. Again, 50,000 DCs were either incubated with or without SEA for 6 hours *in vitro*. Unbound SEA was washed off with PBS and cells were injected into the MLNs of individual recipient animals. 5 days after transfer the injected MLNs were harvested and MLN cells restimulated with SEA *in vitro.* To detect antigen specific immune responses, secreted cytokines were measured in the supernatants of these cultures. Similar to what we had observed after small intestinal DC transfers, lymph DCs that were not loaded with SEA did not induce any immune responses in the recipient MLNs. After being loaded with SEA, lymph DCs did induce antigen specific immune responses upon transfer, indicated by the secretion of IFN-γ, IL-4, IL-5 and IL-13 in restimulation cultures. In unstimulated conditions minimal amounts of cytokines were produced, whereas after restimulation 4 ng/ml of IFN-γ, 0.4 ng/ml of IL-4, 0.9 ng/ml of IL-5 and 8 ng/ml of IL-13 were detected (Fig. 6-2 C). The concentrations of the released cytokines were comparable with small intestinal DC transfers for IFN-γ and IL-4 but increased for IL-5 and IL-13. We had previously observed that the enzymatic digestion during small intestinal DC isolation negatively affected cell survival *in vitro*, which could explain the reduced responses after small intestinal DC transfer compared to lymph DCs. As previously published with OVA protein (Cerovic et al., 2013) and corresponding with our own observations, lymph DCs retained the potential to take up SEA *in vitro* and present it to naive T cells upon transfer into wild type recipient animals.

Once DCs reach the MLNs they fully mature and present intestinal antigens to naive T cells and can be distinguished from resident DCs by their higher expression of MHCII (Cerovic et al., 2013). To address whether migratory MLN DCs were still able to take up exogenous antigen *in vitro* and present it once transferred into recipient mice, MLNs of C57BL/6 animals were harvested. Tissues were digested with 8 U/ml Liberase and 10 µg/ml DNase, cells stained with fluorescent antibodies and total migratory MLN DCs purified by FACS sorting. 50,000 cells were incubated with or without SEA for 6 hours *in vitro*, washed and transferred under the MLN capsule of recipient animals. The induction of antigen specific immune responses in these lymph nodes was assessed 5 days after transfer by restimulating harvested MLN cells with SEA *in vitro*. Cytokine

production during *in vitro* restimulation was measured in the supernatants by ELISA. As previously observed, DCs that were not loaded with SEA did not induce any immune responses in the recipient MLNs after transfer. In contrast to what we had observed from small intestinal and lymph DCs, SEA-loaded migratory DCs did not induce any antigen specific immune responses upon transfer, as we could not detect any cytokines after restimulation (Fig. 6-2 D). We were confident that viable MLN DCs had been transferred, as this was assessed by Trypan blue exclusion after the incubation period with SEA, and the cell viability was comparable to small intestinal and lymph DCs. Therefore, our results indicate that immature DCs from the small intestine and semi-mature DCs from lymph were able to present *in vitro* loaded SEA and could induce antigen-specific immune responses upon transfer, whereas fully mature migratory DCs from the MLNs were no longer able to present *in vitro* loaded antigen upon transfer.

6.1.2 CD11b-expressing dendritic cells are uniquely able to induce Th2 responses against egg antigens

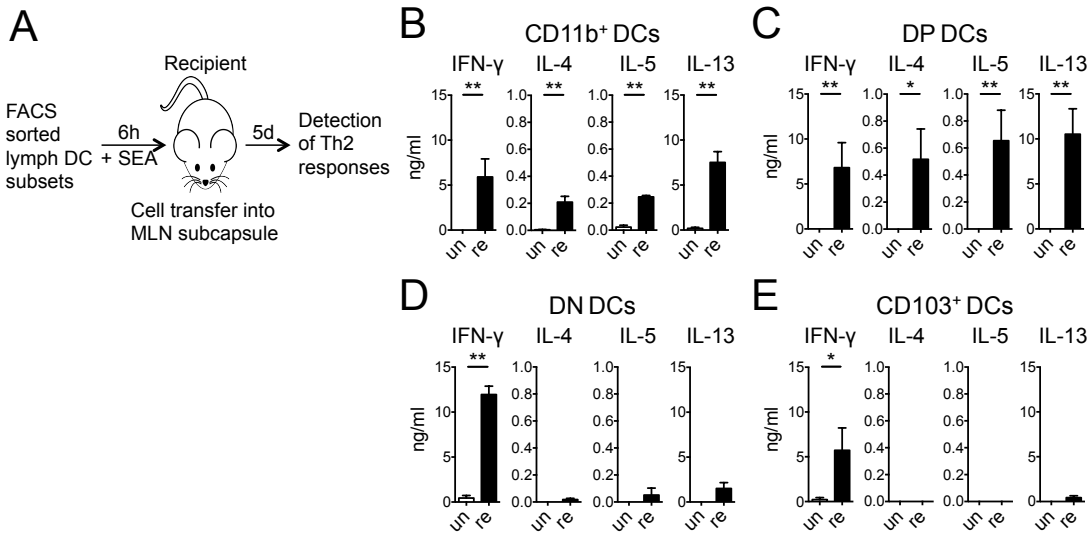

Figure 6-3. Lymph CD11b$^+$ and DP are specialized to induce Th2 responses upon transfer

Lymph DC subsets were purified by FACS sorting, loaded with SEA *in vitro* and transferred under the capsule of recipient mice. 5 days after transfer MLN restimulation cultures were set up to quantify immune responses induced by the transferred cells. A schematic of the experimental protocol is shown in A. Cytokine responses measured from MLNs, from unstimulated (un) and restimulated (re) cultures, in which SEA-loaded CD11b$^+$ (B), DP (C), DN (D) or CD103$^+$ (E) lymph DCs had been transferred. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (*P ≤ 0.05; **P ≤ 0.01).

As we had observed that total small intestinal and lymph migrating DCs were able to present *in vitro* loaded SEA when transferred into wild type recipient mice, we tested whether specific DC subsets were specialized to induce these responses. In our SEA uptake experiments we had discovered that $CD11b^+$ and DP DC subsets transported intestinal egg antigen to the draining lymph nodes and therefore hypothesized that these subsets were also responsible for the induction of Th2 responses. Similarly, $CD11b^+$ and DP DCs were greatly reduced in the MLNs of $IRF4^{f/f}$ CD11c-cre-positive mice, suggesting that they were essential for driving Th2 responses, which were impaired in these animals.

As lymph DCs induced stronger responses after transfer compared to small intestinal DCs, which could be related to the enzymatic digestion necessary to isolate them from the intestine, we collected lymph from cannulated MLNx C57BL/6 mice and individually purified the four DC subsets by FACS sorting. To ensure that egg antigen was equally available to all DCs we incubated equal numbers of each DC subset with 15 µg/ml SEA *in vitro.* 30,000 cells of each subset were incubated with SEA for 6 hours, washed and transferred under the capsule of wild type recipient mice. After 5 days, the injected MLNs were harvested and restimulated with SEA *in vitro* and antigen specific immune responses were analysed by measuring secreted cytokines in the supernatants (Fig. 6-3 A). We did not observe any cytokine secretion in unstimulated wells, indicating that all primed T cell responses required restimulation. In restimulation cultures of MLNs that had received SEA-loaded $CD11b^+$ lymph DCs we measured 5 ng/ml of IFN-γ, 0.2 ng/ml of IL-4, 0.2 ng/ml of IL-5 and 7 ng/ml of IL-13 (Fig. 6-3 B), indicating that SEA-loaded $CD11b^+$ DCs were sufficient to prime Th2 responses after transfer. These cytokines were also measured in MLNs from DP DC recipient animals. The amount of cytokines induced by DP DCs was higher compared to $CD11b^+$ DC recipient mice, especially for the cytokines IL-4, IL-5 and IL-13 (Fig. 6-3 C), suggesting that DP DCs were more efficient in priming antigen-specific Th2 cells. Transferred SEA-loaded DN and $CD103^+$ DCs induced IFN-γ responses, but not Th2 cells, identified by the lack of IL-4, IL-5 and IL-13 in the supernatants of MLN restimulation cultures (Fig. 6-3 D&E). This indicated that DN and $CD103^+$ DCs were viable, presented antigen and induced immune responses, but were not specialized to prime Th2 cells against egg antigens.

Our results therefore indicated that SEA-loaded lymph CD11b^{+} and DP DCs, but not DN and CD103^{+} DCs, were sufficient to induce Th2 responses upon transfer. As these same subsets also carried egg antigen in lymph, and were absent in the MLNs of IRF4$^{f/f}$ CD11c-cre-positive mice, we had thus clearly identified that CD11b^{+} and DP DCs are specialized to induce intestinal Th2 responses.

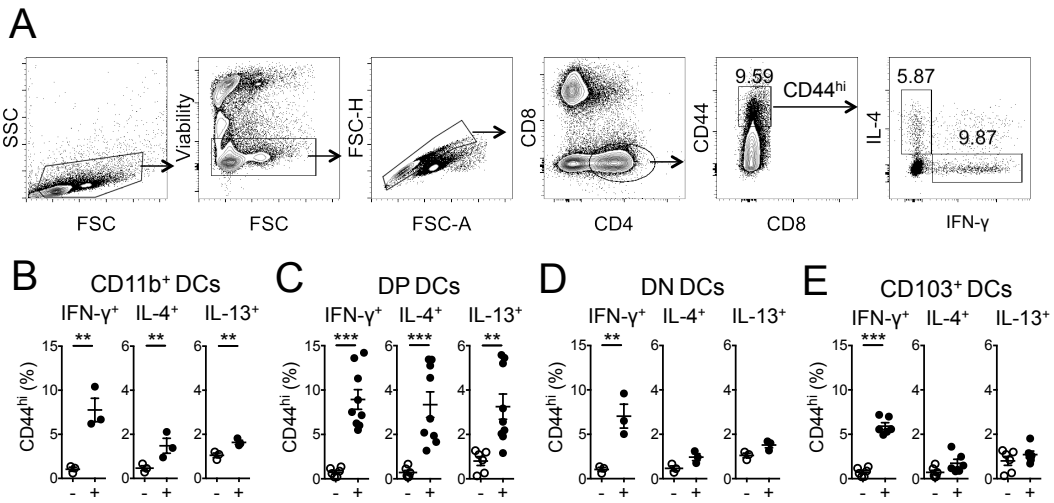

Figure 6-4. The transfer of SEA-loaded CD11b^{+} and DP lymph DCs induced CD44^{hi} CD4 T cell responses in the injected MLNs

Lymph DC subsets were purified by FACS sorting, loaded with SEA *in vitro* and transferred under the capsule of recipient mice. 5 days after transfer injected MLNs were harvested and stimulated with (+) or without (-) PMA/ionomycin. After cell surface marker staining, cell fixation and permeabilization and intracellular staining, cells were analysed by flow cytometry and the percentage of IFN-γ, IL-4 and IL-13 expressing CD44^{hi} CD4 T cells assessed. A representative gating strategy of stimulated DP DCs is shown in A. IFN-γ, IL-4 and IL-13 expressing CD44^{hi} CD4 T cells were compared between unstimulated and stimulated conditions from MLNs, in which SEA-loaded CD11b^{+} (B), DP (C), DN (D) or CD103^{+} (E) lymph DCs had been transferred. Data represent at least two independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied for statistical analysis (**P ≤ 0.01; ***P ≤ 0.001).

We had observed that specific subsets of SEA-loaded lymph DCs were sufficient to prime immune responses after being transferred into recipient MLNs. Using *in vitro* restimulation cultures we had determined that these responses were antigen specific and required restimulation with SEA to secrete cytokines, whereas unstimulated MLN cells did not secrete cytokines *in vitro*. During egg injection we had observed that CD4 T cells were responsible for secreting IFN-γ, IL-4 and IL-13 in response to egg antigens. To confirm that the transfer of DCs induced the same cytokine production by CD4 T cells, we analysed cell specific cytokine secretion by flow cytometry. Lymph DCs were collected from cannulated MLNx C57BL/6

animals and individual DC subsets were purified by FACS sorting. 30,000 cells of each subset were incubated with SEA for 6 hours, washed and transferred under the MLN capsule of individual recipient mice. 5 days after transfer the injected MLNs were harvested and digested with 8 U/ml Liberase and 10 µg/ml DNase. Single cell suspensions were stimulated with or without PMA/ionomycin in the presence of Golgi stop for 4 hours, and cell surface markers stained with fluorescent antibodies. After cell fixation and permeabilization intracellular cytokines were stained and cells analysed by flow cytometry. Live CD4 T cells were selected and activated T cells were identified as $CD44^{hi}$ cells. The expression of IFN-γ, IL-4 and IL-13 was analysed and compared between unstimulated and PMA/ionomycin stimulated cells (Fig. 6-4 A). In general, less than 1% of $CD44^{hi}$ cells expressed IFN-γ, IL-4 and IL-13 in unstimulated conditions, indicating that PMA/ionomycin stimulation was needed to drive expression of these cytokines *in vitro*. In MLNs harvested from SEA-loaded $CD11b^{+}$ DCs recipients, 8% of $CD44^{hi}$ CD4 T cells expressed IFN-γ and 2% expressed IL-4 and IL-13 after PMA/ionomycin stimulation (Fig. 6-4 B). As these percentages were significantly higher compared to the unstimulated conditions we could confirm that IFN-γ, IL-4 and IL-13 were indeed produced by activated CD4 T cells in MLNs harvested from SEA-loaded $CD11b^{+}$ DC recipients. In fact, the majority of cytokines were produced by $CD44^{hi}$ and not by $CD44^{low}$ cells, indicating that CD4 T cells of an activated effector/memory phenotype responded to egg antigens. Furthermore, IFN-γ-producing cells did not co-express IL-4 or IL-13, whereas the populations of IL-4- and IL-13-producing cells overlapped, indicating that IFN-γ-producing cells and Th2 cells were primed against egg antigens.

Expression of IFN-γ, IL-4 and IL-13 was even more pronounced in MLNs harvested from SEA-loaded DP DC recipients, with 9% of $CD44^{hi}$ CD4 T cells expressing IFN-γ, and 3.5% expressing IL-4 and IL-13 after PMA/ionomycin stimulation (Fig. 6-4 C). The stronger immune responses observed after the transfer of SEA-loaded DP DCs compared to $CD11b^{+}$ DCs corresponded with our previous findings in *in vitro* restimulation cultures, which showed the same trend. Intracellular staining from MLNs of SEA-loaded DN and $CD103^{+}$ DCs revealed that IFN-γ producing $CD44^{hi}$ CD4 T cells could be identified after PMA/ionomycin stimulation, but that the percentage of IL-4 and IL-13 expressing cells was similar to background levels measured without stimulation (Fig. 6-4 D&E). This matched our previous observations in restimulation cultures, which showed that the transfer

of SEA-loaded DN and CD103^{+} DCs induced antigen specific IFN-γ responses but no production of IL-4, IL-5 or IL-13. Therefore, our findings confirmed that the transfer of SEA-loaded lymph CD11b^{+} and DP DC subsets induced IFN-γ and Th2 responses in the recipient MLNs, which were produced by individual populations of activated CD44^{hi} CD4 T cells.

6.1.3 IL-4 and IRF4 are not required by CD11b-expressing dendritic cells to induce Th2 responses

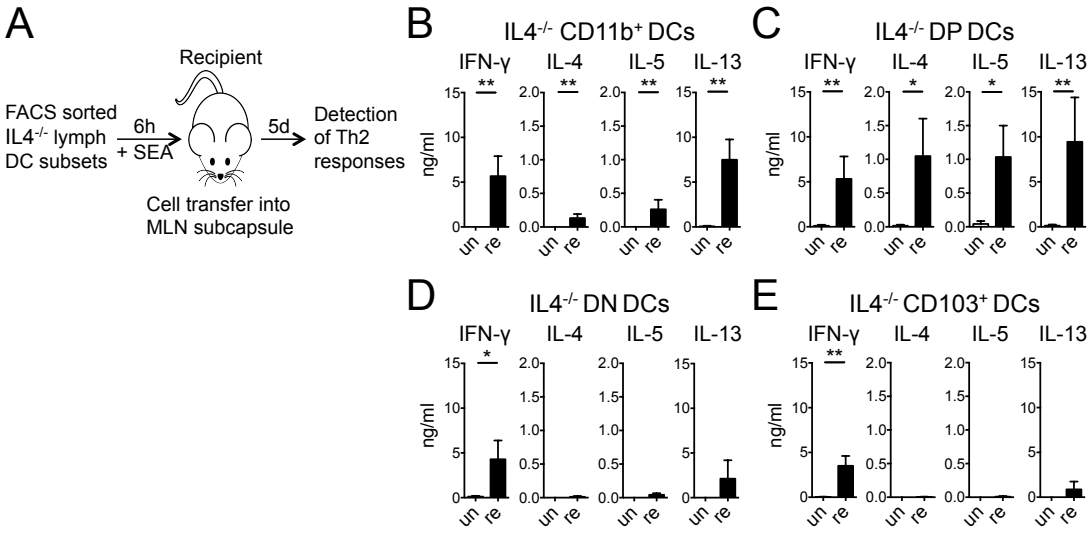

Figure 6-5. IL-4 expression by DCs is not required for the induction of Th2 responses after DC transfer into recipient mice

Lymph DC subsets from MLNx IL-4$^{-/-}$ mice were purified by FACS sorting, loaded with SEA *in vitro* and transferred under the capsule of recipient mice. The induced immune response was measured in restimulation cultures 5 days after transfer. The experimental protocol is illustrated in A. Immune responses after transfer of IL-4$^{-/-}$ SEA-loaded CD11b^{+} (B), DP (C), DN (D) or CD103^{+} (E) lymph DCs were analysed in unstimulated (un) and restimulated (re) conditions. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (*P ≤ 0.05; **P ≤ 0.01).

As we had observed that transferred SEA-loaded lymph DCs were sufficient to induce Th2 responses in the recipient MLNs, these DCs were directly able to prime Th2 cells. The priming of Th2 cells has been a controversial topic in the literature. Th2 cells require IL-4 for their differentiation *in vitro* and Th2 responses do not develop in the absence of IL-4 or IL-4 receptor signalling (Jankovic et al., 2000), which we had also observed in IL-4$^{-/-}$ mice after egg injection. However, it has been demonstrated that IL-4$^{-/-}$ BMDCs induce similar Th2 responses as their

wild type counterparts (Maldonado-López et al., 2001; MacDonald and Pearce, 2002) and other cells like basophils, eosinophils or ILC2s have therefore been suggested to produce initial IL-4, required for Th2 differentiation. However, it has also been shown that naive CD4 T cells produce IL-4 in low amounts upon TCR activation *in vitro* (Yamane et al., 2005), suggesting that Th2 differentiation does not require exogenous IL-4.

As basophils, eosinophils and ILC2s mostly reside within the intestinal lamina propria and not within the lymph nodes, it was unclear which cells produced the initial IL-4 required for Th2 differentiation upon DC transfer. To address whether IL-4 was produced by the transferred DCs and required for the induction of Th2 responses in the injected MLNs, we cannulated MLNx IL-4$^{-/-}$ mice. Thoracic duct lymph was collected for 18 hours and DC subsets purified by FACS sorting. 30,000 cells of each subset were incubated with 15 µg/ml SEA *in vitro*. After 6 hours any unbound SEA was washed off and cells were transferred under the MLN capsule of individual wild type recipient mice. 5 days after transfer the injected MLNs were harvested and restimulated with SEA for 3 days *in vitro* and the antigen-specific production of cytokines was measured in the supernatants (Fig. 6-5 A). We observed that the transfer of SEA-loaded IL-4$^{-/-}$ CD11b^{+} DCs induced both IFN-γ and Th2 cytokines, with 5 ng/ml of IFN-γ, 0.2 ng/ml of IL-4, 0.3 ng/ml of IL-5 and 7 ng/ml of IL-13 being secreted after restimulation (Fig. 6-5 B). The transfer of SEA-loaded IL-4$^{-/-}$ DP DCs induced even stronger responses in the MLN, revealed by the increased amounts of IL-4, IL-5 and IL-13 detected after restimulation with SEA (Fig. 6-5 C). Similar to what we had observed from SEA-loaded C57BL/6 DC subsets, the transfer of SEA-loaded IL-4$^{-/-}$ DN and CD103^{+} DCs only induced antigen specific IFN-γ responses, but no production of IL-4, IL-5 or IL-13 (Fig. 6-5 D&E). Therefore, the transfer of SEA-loaded IL-4$^{-/-}$ DC subsets resulted in the induction of very similar immune responses compared to C57BL/6 DCs, with CD11b^{+} and DP DCs being uniquely able to prime Th2 response upon transfer. Therefore, IL-4 expression by tissue-derived DCs was not required for the induction of Th2 responses *in vivo*, confirming similar findings using IL-4$^{-/-}$ BMDCs (Maldonado-López et al., 2001; MacDonald and Pearce, 2002). We therefore suggest that exogenous IL-4 is not required to induce Th2 responses in our system of DC transfer into the MLNs but that a small amount of naive T cells produce the initial IL-4 that is necessary for Th2 differentiation, as previously suggested by Yamane *et al.* (Yamane et al., 2005).

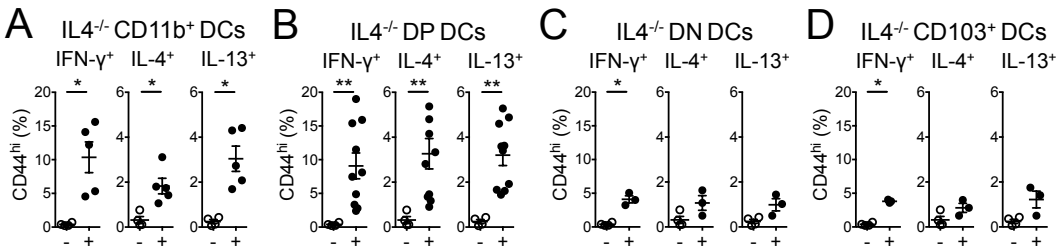

Figure 6-6. SEA-loaded IL-4$^{-/-}$ DCs are not deficient in inducing CD44^{hi} CD4 T cell responses upon transfer into recipient mice

Lymph DC subsets from MLNx IL-4$^{-/-}$ mice were purified by FACS sorting, loaded with SEA *in vitro* and transferred under the capsule of recipient mice. 5 days after transfer injected MLNs were harvested and stimulated with (+) or without (-) PMA/ionomycin. Cell surface markers were stained, cells fixed and permeabilized and cytokines stained intracellularly. The percentage of IFN-γ, IL-4 and IL-13 expressing CD44^{hi} CD4 T cells was then assessed by flow cytometry and compared between unstimulated and stimulated conditions. MLNs, in which SEA-loaded IL-4$^{-/-}$ CD11b^{+} (B), DP (C), DN (D) or CD103^{+} (E) lymph DCs had been transferred were analysed. Data represent at least two independent experiments (mean ± SEM) with each point representing one animal. Mann-Whitney *U* tests were applied for statistical analysis (*P ≤ 0.05; **P ≤ 0.01).

We had observed that IL-4$^{-/-}$ DCs, when loaded with SEA *in vitro* and transferred under the capsule of recipient mice, were as effective in inducing antigen specific cytokine responses as their C57BL/6 counterparts. To confirm that these cytokine responses were produced by activated T cells, we analysed the recipient MLNs of IL-4$^{-/-}$ DC transfers by flow cytometry. To obtain IL-4$^{-/-}$ lymph DCs, thoracic duct lymph was collected from cannulated MLNx IL-4-/- animals for 18 hours and cells stained for flow cytometry. DC subsets were purified by FACS sorting and 30,000 cells of each subset incubated with SEA *in vitro.* Unbound SEA was then washed off and cells transferred into the MLNs of recipient animals. MLNs were harvested 5 days after transfer and digested with DNase and Liberase. Cell suspensions were stimulated with or without PMA/ionomycin for 4 hours in the presence of Golgi Stop solution and then stained with fluorescent antibodies for cell surface markers. Cells were then fixed and permeabilized, after which intracellular cytokines were stained with fluorescent monoclonal antibodies. Then the expression of IFN-γ, IL-4, and IL-13 on live CD44^{hi} CD4 T cells was assessed by flow cytometry. In general, the background expression of these cytokines was less than 1%, which we measured in unstimulated conditions. PMA/ionomycin stimulated MLNs from SEA-loaded IL-4$^{-/-}$ CD11b^{+} and DP DC recipient animals showed that IFN-γ, IL-4, and IL-13 producing CD4 T cells had been induced.

$CD44^{hi}$ and not $CD44^{low}$ CD4 T cells produced the majority of cytokines and around 10% of $CD44^{hi}$ CD4 T cells were IFN-γ^{+}, and 2-3% were IL-4^{+} or IL-13^{+}. We also noticed that the transfer of DP DCs induced stronger immune responses than $CD11b^{+}$ DCs, a phenomenon that we had also observed in previous experiments (Fig. 6-6 A&B). The induction of immune responses in MLNs from SEA-loaded IL-$4^{-/-}$ DN and $CD103^{+}$ DC recipient animals was relatively low. 5% of $CD44^{hi}$ CD4 T cells expressed IFN-γ, whereas only 1-2% expressed IL-4 or IL-13 after PMA/ionomycin stimulation. Thus, only the expression of IFN-γ, but not the expression of IL-4 and IL-13, was significantly different from unstimulated conditions (Fig. 6-6 C&D). Therefore, these results corresponded with our *in vitro* restimulation data where IL-$4^{-/-}$ DN and $CD103^{+}$ DCs only induced antigen specific IFN-γ responses. Taken together, intracellular staining for IFN-γ, IL-4 and IL-13 confirmed that the transfer of SEA-loaded IL-$4^{-/-}$ $CD11b^{+}$ and DP DCs induced the production of IFN-γ, IL-4 and IL-13 in activated $CD44^{hi}$ CD4 T cells, similar to what we had observed in our C57BL/6 DC transfer experiments. Together, these data conclusively showed that IL-4 deficiency in DCs did not affect their ability to prime egg antigen specific T cell responses, and had no affect on the induction of Th2 cells by these cells.

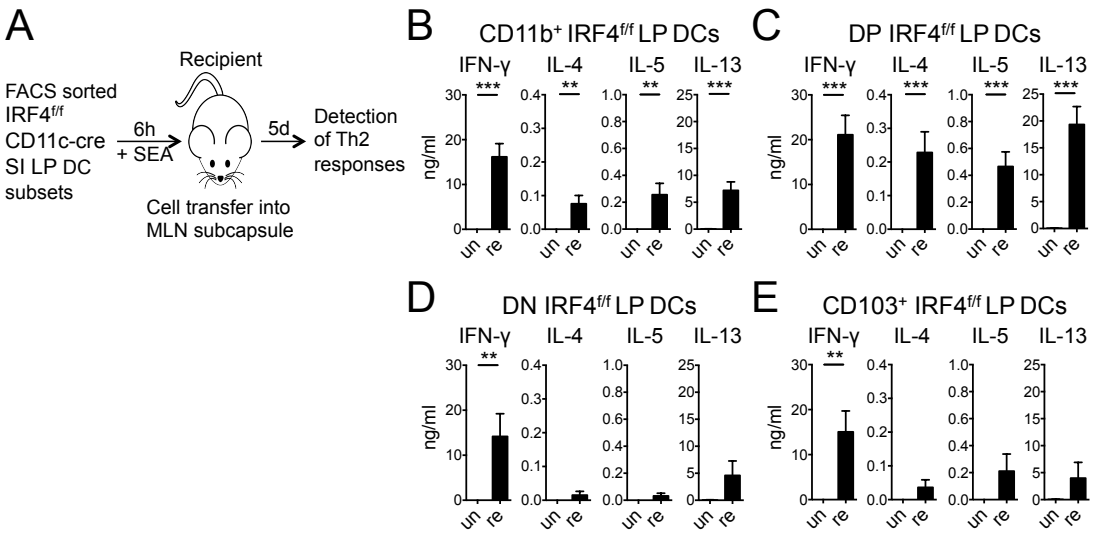

Figure 6-7. SEA-loaded IRF4$^{f/f}$ CD11c-cre-positive DC subsets retain their potential to induce Th2 responses if transferred into the MLNs of recipient mice

Small intestinal LP DC subsets were FACS purified from IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras and loaded with SEA *in vitro.* 30,000 cells were transferred into individual recipient mice where the induced immune responses were measured in MLN restimulation cultures 5 days after transfer. A schematic of the experimental protocol is shown in A. Immune responses after transfer of IRF4$^{f/f}$ CD11c-cre-positive SEA-loaded CD11b^{+} (B), DP (C), DN (D) or CD103^{+} (E) SI LP DCs were analysed in unstimulated (un) and restimulated (re) conditions and the released cytokines measured by ELISA. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Mann-Whitney *U* tests were applied for statistical analysis (**P ≤ 0.01; ***P ≤ 0.001).

Another important question that we intended to address by DC transfer was if IRF4 deficiency in DCs affected their capacity to induce egg antigen specific T cell responses, in particular their ability to prime Th2 cells.

The injection of *S. mansoni* eggs into the intestines of IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras induced IFN-γ, but no Th2 responses, suggesting that the lack of IRF4 affected DC functionality in priming Th2 cells. We observed that IRF4 deficiency in DCs led to differences in DC subsets compositions both in the lamina propria and the MLNs. DP DCs decreased in the intestinal lamina propria, whereas in the MLNs CD11b^{+} DCs were reduced by 50% and DP DCs almost completely absent. However, uptake of egg antigen was not impaired in the lamina propria of IRF4$^{f/f}$ CD11c-cre-positive mice but SEA-transporting DCs were greatly decreased in the MLNs. We therefore hypothesized that the ineffective Th2 immune response upon injection of *S. mansoni* eggs into the intestine, was influenced by the lack of antigen carrying CD11b^{+} and DP DCs in the MLNs.

This hypothesis was supported by our finding that antigen presentation of OVA to OT-II CD4 T cells was not impaired in the remaining MLN DCs from IRF4$^{f/f}$ CD11c-cre-positive animals, which suggested that IRF4-deficient DCs could present antigen and prime naive T cells. To furthermore show that IRF4-deficient DCs were functionally capable of inducing Th2 responses, intestinal DCs from IRF4$^{f/f}$ CD11c-cre-positive mice were loaded with SEA *in vitro* and transferred into wild type recipient mice. We had previously demonstrated that antigen uptake by intestinal DCs was not affected by the deletion of IRF4 and that intestinal SEA-loaded C57BL/6 DCs were capable of inducing Th2 responses upon transfer. Thus, the transfer of SEA-loaded IRF4-deficient DCs would directly determine if their Th2 inducing potential was impaired or if their absence in the draining lymph nodes was responsible for the impaired Th2 responses observed in IRF4$^{f/f}$ CD11c-cre-positive mice.

Small intestines of IRF4$^{f/f}$ CD11c-cre-positive bone marrow chimeras were enzymatically digested with Collagenase VIII and single cell suspensions stained for flow cytometry. Individual intestinal DC subsets were purified by FACS sorting and 30,000 cells of each subset were incubated with SEA *in vitro*. After 6 hours unbound antigen was washed off and DCs were injected under the MLN capsule of wild type recipient animals. After 5 days the injected MLNs were harvested and restimulated with SEA *in vitro* for 3 days. To determine the induction of antigen-specific immune responses induced by the transferred DCs, produced cytokines were measured in the supernatants by ELISA (Fig. 6-7 A). Upon transfer of SEA-loaded CD11b^{+} DCs we detected IFN-γ, IL-4, IL-5 and IL-13 after restimulation. 15 ng/ml of IFN-γ, 0.1 ng/ml of IL-4, 0.2 ng/ml of IL-5 and 7 ng/ml of IL-13 were measured, indicating that antigen specific IFN-γ and Th2 responses were induced by IRF4-deficient DCs (Fig. 6-7 B). Cytokine levels from DP DC transferred MLNs were higher, reaching 20 ng/ml for IFN-γ, 0.2 ng/ml for IL-4, 0.5 ng/ml for IL-5 and 18 ng/ml for IL-13 (Fig. 6-7 C). This indicated that SEA- loaded DP DCs from IRF4$^{f/f}$ CD11c-cre-positive mice were able to induce stronger IFN-γ and Th2 responses than CD11b^{+} DCs when transferred into recipient mice, with matched our previous observations from C57BL/6 mice. Similar to what we had observed with SEA-loaded C57BL/6 DCs, DN and CD103^{+} DC subsets from IRF4$^{f/f}$ CD11c-cre-positive mice induced strong IFN-γ responses but only minor levels of Th2 cytokines after being transferred into recipient MLNs (Fig. 6-7 D&E). Therefore, the deletion of IRF4 in DCs did not inhibit antigen presentation and T cell priming,

which was consistent with our findings that the presentation of OVA to OT-II cells was also not inhibited in these cells. Furthermore, IRF4 deletion did not affect the potential of DCs to prime Th2 responses after transfer. Similar to C57BL/6 DCs, $IRF4^{f/f}$ CD11c-cre-positive DP and $CD11b^{+}$ DC subsets remained specialized for the induction of Th2 responses, with small intestinal DP DCs being the most potent Th2-inducing subset. Thus, in our model of intestinal *S. mansoni* egg immunization IRF4 deficiency did not alter DCs functionality but rather reduced $CD11b^{+}$ and DP DC numbers and antigen availability in the draining lymph nodes, which likely impaired Th2 cell priming.

6.1.4 Distinct subsets of CD11b-expressing dendritic cells drive Th2 responses in the small intestine and colon

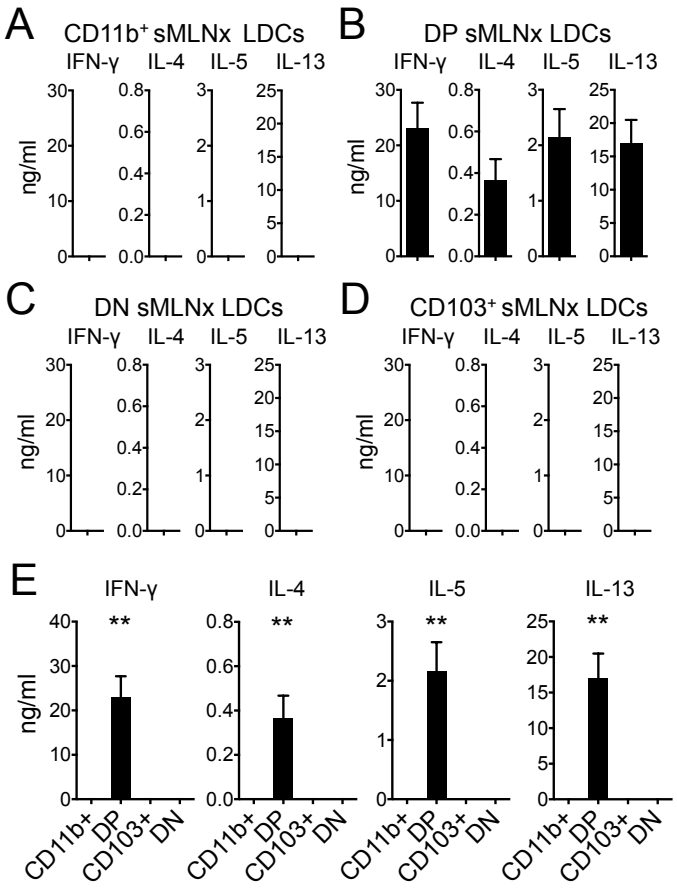

Figure 6-8. Lymph migrating DP DCs from the small intestine are uniquely specialized to present egg antigen to naive T cells and induce Th2 responses upon transfer

DC subsets were FACS purified from the lymph of sMLNx C57BL/6 mice after small intestinal egg injection. Individual DC subsets were injected under the capsule of recipient mice and the induced immune responses assessed after 5 days in restimulation cultures. Secreted cytokine levels after restimulation are shown for MLNs where CD11b$^+$ (A), DP (B), DN (C) and CD103$^+$ DCs had been transferred. Immune responses induced by the transferred DC subsets were compared in E. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Kruskal-Wallis tests were applied for statistical analysis in E (***P ≤ 0.001).

Our previous data indicated that SEA-loaded CD11b$^+$ and DP DCs from C57BL/6 mice were specialized to induce antigen specific Th2 responses when transferred into wild type recipient animals. Hereby, equal numbers of cells were collected from the lymph of MLNx animals, loaded with SEA *in vitro* and transferred under the MLN capsule of recipient animals. We observed that lymph DP DCs induced the strongest immune responses, followed by CD11b$^+$ DCs; whereas DN and CD103$^+$ DCs only induced IFN-γ but no Th2 responses.

To investigate if in the physiological context of intestinal egg injection the same subsets were involved in Th2 priming in the MLNs, lymph DC subsets were directly collected from egg-injected donor mice and transferred into recipient animals. Lymph was collected from sMLNx donor mice after small intestinal egg injection, which allowed the selective collection of small intestinal migrating DCs. Lymph was collected for 18 hours, then cells were stained with fluorescent monoclonal antibodies and DC subsets were purified by FACS sorting. 30,000 DCs of each subset were resuspended in 5 µl PBS and directly transferred under the MLN capsule of recipient animals. 5 days after transfer the injected MLNs were harvested and MLN cells were restimulated with SEA for 3 days *in vitro* and antigen specific immune responses induced by the transferred DCs were analysed by measuring the secreted cytokines. Contrary to our SEA-loading experiments, we observed that the transfer of lymph $CD11b^+$ DCs from egg injected donor mice induced no antigen specific immune responses in the recipient animals (Fig. 6-8 A). DP DCs however induced robust immune responses, measured by the secretion of IFN-γ, IL-4, IL-5 and IL-13 after restimulation (Fig. 6-8 B). Furthermore, no antigen specific immune responses were detected in the lymph nodes of DN and $CD103^+$ DC injected animals (Fig. 6-8 C&D). This indicated that small intestinal DP DCs were solely responsible for priming egg antigen specific T cells when eggs were injected into the small intestine. As our previous data showed that lymph DP DCs were the most numerous subset to carry labelled SEA from the small intestine, it is likely that the other DC subsets did not carry sufficient amounts of antigen to induce T cell responses upon transfer and this could explain why the other lymph DC subsets did not induce any antigen specific immune responses. Thus, lymph migratory DP DCs were solely responsible to present small intestinal egg antigen to naive T cells under physiological conditions and induced antigen specific Th2 responses when transferred into wild type recipient animals (Fig. 6-8 E).

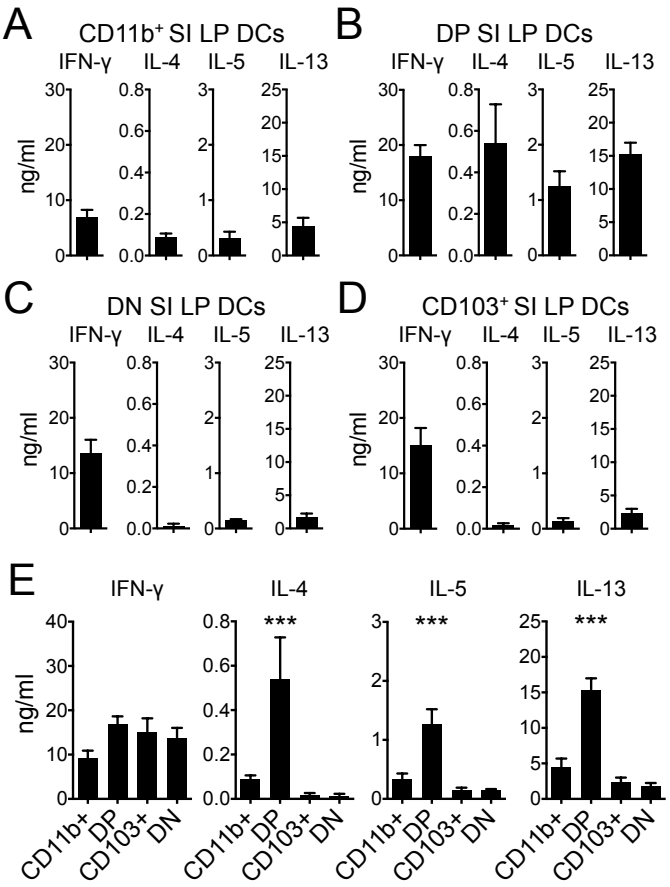

Figure 6-9. SEA-loaded small intestinal DP DCs induce strong Th2 responses when transferred into recipient mice

CD11b and CD103 expressing DC subsets were FACS purified from enzymatically-digested small intestines and loaded with SEA *in vitro*. 30,000 cells of each subset were transferred under the MLN capsule of recipient mice and induced immune responses analysed in restimulation cultures 5 days after transfer. Secreted cytokine levels after restimulation are shown for MLNs where SEA-loaded $CD11b^+$ (A), DP (B), DN (C) and $CD103^+$ SI LP DCs had been transferred. Immune responses induced by the transferred DC subsets were compared in E. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Kruskal-Wallis tests were applied for statistical analysis in E (***$P \leq 0.001$).

We had observed that lymph migrating DCs from egg-injected small intestines were uniquely able to induce Th2 responses upon transfer and under these conditions neither $CD11b^+$, DN nor $CD103^+$ DCs induced any antigen-specific immune responses. We had also observed that DP DCs were the most numerous subset to carry small intestinally-injected fluorescently labelled SEA in lymph and only small numbers of $CD11b^+$, DN and $CD103^+$ DCs were involved in this process. To clarify whether the physiological uptake of antigen or cell intrinsic properties of antigen processing and presentation predisposed DP DCs from the small intestine to be the only subset to induce antigen-specific immune responses after transfer we incubated all DC subsets with SEA *in vitro.* As we had observed

that DC specific differences existed between the small intestine and colon we isolated DCs from the lamina propria of the small intestine or colon, as not enough cells could be collected from cMLNx lymph or colon-draining cMLNs to allow a direct comparison. Thus, small intestines of C57BL/6 mice were enzymatically digested with Collagenase VIII and single cell suspensions stained for flow cytometry. DC subsets were purified by FACS sorting and incubated with 15 µg/ml SEA for 6 hours *in vitro*. Unbound SEA was washed off and 30,000 cells of each subset transferred under the MLN capsule of recipient mice. 5 days after transfer, MLNs were harvested and restimulated with SEA *in vitro* and the immune responses induced by the individual transferred DC subsets were assessed by the secretion of cytokines in response to SEA restimulation.

The transfer of SEA-loaded small intestinal $CD11b^+$ DCs induced weak IFN-γ and Th2 responses in the recipient mice, with 8 ng/ml of IFN-γ, 0.1 ng/ml IL-4, 0.2 ng/ml IL-5 and 5 ng/ml IL-13 detected in restimulation cultures (Fig. 6-9 A). Antigen-specific immune responses induced by SEA-loaded DP DCs after transfer were higher and cytokine secretion reached 18 ng/ml for IFN-γ, 0.5 ng/ml for IL-4, 1.2 ng/ml for IL-5 and 15 ng/ml for IL-13 (Fig. 6-9 B). Transferred SEA-loaded DN and $CD103^+$ DCs induced IFN-γ responses of around 15 ng/ml but no significant amount of Th2 cytokines (Fig. 6-9 C&D). These findings corresponded with our data from SEA-loaded lymph DCs where DN and $CD103^+$ subsets induced IFN-γ but no Th2 responses. When we compared the immune responses induced by the different transferred SEA-loaded DC subsets it became again apparent that small intestinal DP DCs were specialized to induce Th2 responses, whereas IFN-γ responses could be induced by all subsets (Fig. 6-9 E). This suggested that DP DCs from the small intestine were inherently better at either processing and presenting egg antigens or priming Th2 cells compared to the other subsets. This ability, together with our previous findings that they were the most numerous subset to transport egg antigen from the small intestine to the MLNs, could explain why they are the primary DC subset to induce Th2 responses in the small intestine.

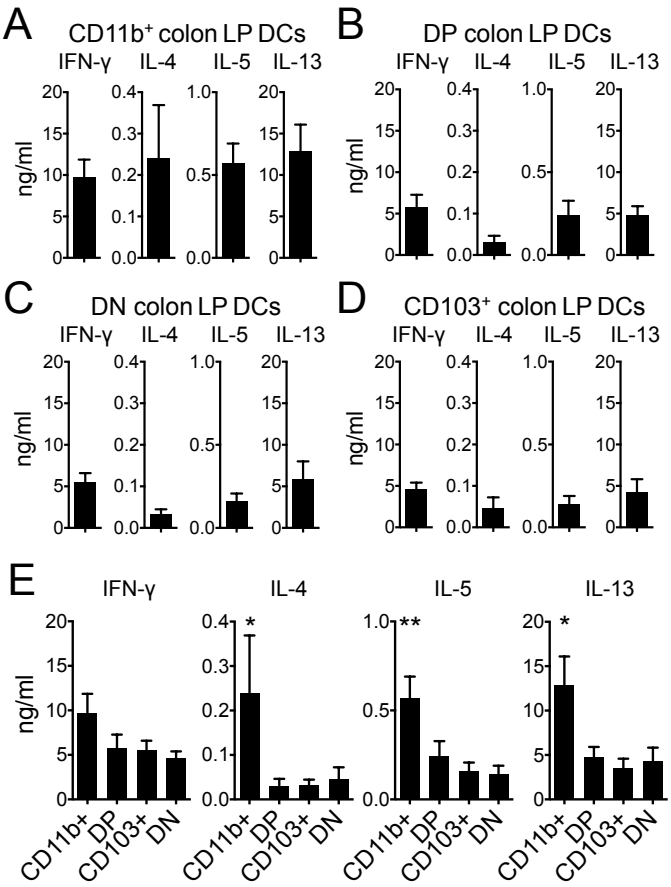

Figure 6-10. SEA-loaded colon-derived CD11b$^+$ DCs and not DP DCs are specialized to induce Th2 responses upon transfer

DC subsets were FACS purified from enzymatically-digested colons of C57BL/6 mice and loaded with SEA *in vitro*. Individual subsets were transferred under the MLN capsule of recipient mice and 5 days after transfer MLN restimulation cultures were set up. Secreted cytokine levels after restimulation are shown for MLNs where SEA-loaded CD11b$^+$ (A), DP (B), DN (C) and CD103$^+$ colon LP DCs had been transferred. Cytokine levels from all DC subset transfers were compared in E. Data represent at least three independent experiments (mean ± SEM) with at least three animals per group. Kruskal-Wallis tests were applied for statistical analysis in E (*P ≤ 0.05; **P ≤ 0.01).

In contrast to the small intestine, labelled SEA injected into the colonic lamina propria was transported to the cMLNs by CD11b$^+$, which led us to hypothesize that this subset might be specialized to induce Th2 responses in the colonic draining lymph nodes. Unfortunately, due to the 10-fold lower numbers of DCs migrating from the colon compared to the small intestine, it was not possible to collect enough DCs from the lymph of colon-injected cMLNx mice to transfer into recipient animals. As an alternative approach, that would allow the direct comparison between small intestinal and colonic DCs, we isolated DCs from the lamina propria, incubated them with SEA *in vitro* and subcapsularly transferred these LP-derived DCs into recipient animals. Thus, colons from C57BL/6 mice were

enzymatically digested and colonic DC subsets purified by FACS sorting. Equivalent to our experiments using small intestinal DCs, 30,000 cells of each subset were incubated with 15 µg/ml SEA for 6 hours, washed and transferred under the MLN capsule of recipient animals. 5 days after transfer MLNs were harvested and restimulated with SEA *in vitro*. After 3 days of restimulation, supernatants were assessed for secreted cytokines. Consistent with what we had observed in previous experiments, the immune responses induced by colonic DCs were generally lower compared to their small intestinal counterparts. In restimulation cultures of transferred SEA-loaded colonic $CD11b^+$ DC MLNs we measured 8 ng/ml of IFN-γ, 0.15 ng/ml of IL-4, 0.2 ng/ml of IL-5 and 7 ng/ml of IL-13 (Fig. 6-10 A). Immune responses in transferred DP, DN and $CD103^+$ DC MLNs were lower, showing secretion of around 5 ng/ml IFN-γ and IL-13 but only minimal amounts of IL-4 and IL-5 (Fig. 6-10 B-D). Statistical comparison between the immune responses induced by the transferred SEA-loaded colonic DC subsets revealed that $CD11b^+$ DCs induced the strongest Th2 responses, whereas IFN-γ responses could be induced by all subsets (Fig. 6-10 E). Together with our observation that colonic $CD11b^+$ DCs were the only subset to transport egg antigen in lymph, these results demonstrated that in contrast to the small intestine, $CD11b^+$ DCs and not DP DCs were specialized to induce Th2 responses in the colon. Thus, we have not only revealed that specific DC subsets are specialized to induce Th2 responses against *S. mansoni* eggs in the intestine but have also discovered that different cellular mechanisms exist between small intestinal and colonic DC subsets, which drive these responses.

6.2 Discussion

When we injected fluorescently labelled SEA into the intestine we observed that both B cells and dendritic cells transported egg antigen in lymph. By transferring immune cells from egg-injected animals under the MLN capsule of recipient mice we were able to directly assess the priming capabilities of these cells *in vivo*. This method of cell delivery into the MLN has recently been developed in our laboratory and has major advantages over other cell transfer systems like intravenous or intraperitoneal transfer (Cerovic et al., 2015). As lymph migrating cells enter the MLN through the MLN capsule, subcapsular injections transfer cells to their biological point of entry, which allows their functional assessment in their physiological environment. Furthermore, the point of cell transfer and entry is exactly known, which allows us to monitor the responses in a precise manner by harvesting only the injected lymph node. Furthermore, subcapsular injections allow the transfer of much smaller cell numbers ranging from 30,000 to 200,000 cells compared to other techniques, which require millions of cells, and makes the transfer and study of tissue-derived cells feasible.

Using subcapsular cell transfers we could directly monitor the T cell priming capabilities of B cells and DCs and observed that lymph DCs but not B cells from egg injected donors were sufficient to induce antigen specific immune responses in the recipient animals (Fig. 6-1). This observation supported many of our previous data, which indicated that $IRF4^+$ CD11c-expressing cells, which includes DCs but not B cells, were required to drive Th2 responses against *S. mansoni* eggs and verified reports from the literature that have shown that the deletion of DCs in CD11c-DTR mice impaired Th2 responses in models of asthma and alum/OVA immunization, as well as during *Schistosoma mansoni* and *Nippostrongylus brasiliensis* infection (van Rijt et al., 2005; Kool et al., 2008; Phythian-Adams et al., 2010; Smith et al., 2011). However, the deletion of DCs in these models did not allow the authors to determine, whether DCs were essential for the induction, maintenance or restimulation of the response. Using our cell transfer system we could clarify that DCs were essential for the induction of T cell responses against *S. mansoni* eggs. Furthermore, they were sufficient to induce these responses upon transfer, suggesting that DCs alone give all the required signals to prime Th2 responses. One of these required signals is the presentation

of antigen via MHCII and we showed that MHCII-deficient DCs were no longer capable of inducing T cell responses after transfer.

Although B cells transported fluorescent antigen in lymph, they could not induce antigen-specific immune responses after transfer, which suggested that they did not directly play a role in T cell priming. However, as demonstrated in the literature, B cells can influence Th2 responses by magnifying the response through antibody secretion, such as IgE, or antigen-presentation to primed activated T cells (León et al., 2014). As our project focused on the identification of the cells responsible for inducing Th2 responses, we did not perform further experiments to assess the supportive role of B cells during the maintenance of the response.

We had identified that flow-sorted DCs were sufficient to prime IFN-γ secreting cells and induce Th2 responses upon transfer. As these cells were sorted from egg injected donor mice, antigen was hereby acquired in the small intestine. However, antigen could also be provided *in vitro* and SEA-loaded FACS purified small intestinal and lymph dendritic cells could induce antigen-specific immune responses upon transfer (Fig. 6-2). As small intestinal DCs represent immature peripheral DCs they are responsible for taking up antigen in the lamina propria, which likely enabled them to also sample antigen *in vitro*. Semi-mature lymph DCs have also been reported to retain the potential to take up soluble antigen and drive T cell differentiation upon *in vitro* stimulation (Cerovic et al., 2013), which was in agreement with our own observations that they could present *in vitro*-loaded SEA. Fully mature DCs isolated from the MLN however could no longer present antigen acquired *in vitro*.

Similar observations had been made early on in the Steinman laboratory. Freshly isolated immature Langerhans cells drove weak T cell responses *in vitro* immediately after isolation but after 3 days of maturation developed into potent T cell stimulators (Schuler and Steinman, 1985). They identified that during maturation antigen was processed and presented via MHCII (Romani et al., 1989). Other studies further demonstrated that MHCII-peptide complexes are present on the cell surface for several days and that mature DCs can acquire novel antigen but no longer process and present it on the cell surface (Villadangos et al., 2005). Thus, immature and semi-mature DCs readily acquire antigen and can process

and present it to naive T cells, which in our case happened during the 5 day immunization period after cell transfer, whereas mature DCs are specialized for the presentation of already acquired antigen over long periods of time and do no longer present novel antigen.

Having identified that DCs primed T cells against egg antigens we furthermore addressed whether certain DC subsets were specialized to induce Th2 responses upon transfer. To exclude any possible bias of antigen uptake, which we had observed during fluorescent SEA injection, we FACS sorted individual DC subsets from lymph of MLNx C57BL/6 mice and incubated them with SEA *in vitro.* Transfer of SEA-loaded lymph DC subsets revealed that $CD11b^+$ and DP DCs were able to induce IFN-γ and Th2 responses upon transfer, whereas DN and $CD103^+$ DCs did induce IFN-γ but not Th2 responses. The induced immune responses from $CD11b^+$ and DP DCs closely resembled those induced directly by injected eggs, which we assessed by cytokine secretion after *in vitro* restimulation with SEA and during PMA/ionomycin stimulation followed by intracellular staining (Fig. 6-3; 6-4). We had therefore demonstrated that CD11b-expressing DCs were specialized to drive Th2 responses against *S. mansoni* egg antigen in the MLNs and induced egg-equivalent immune responses, whereas antigen-specific IFN-γ responses could be induced by all DC subsets.

We furthermore observed that the transfer of DP DCs induced more potent immune responses, compared to $CD11b^+$ DCs, which could play an important role under the physiological conditions of egg injection. As we had observed that DP DCs transported the majority of egg antigen in lymph of small intestinally injected animals, they might therefore represent the most important subset to drive Th2 responses in the small intestine. Conversely, $CD11b^+$ DCs are the most abundant subset in the colon and transported fluorescent antigen in colon injected animals, which could predispose them to drive Th2 responses in the colon.
Interestingly, the transfer of $CD103^+$ and DN DCs induced antigen-specific IFN-γ but no Th2 responses. These results demonstrated that they were able to take up, process and present egg antigens to naive T cells and induce IFN-γ responses but were not able to prime Th2 cells. This observation supports the hypothesis that different DC subsets are specialized to drive distinct immune responses. For example, intestinal DP DCs have been shown to drive Th1, Th17 and Treg responses (Fujimoto et al., 2011; Persson et al., 2013b; Coombes et al., 2007),

whereas $CD103^+$ DCs are specialized to induce Th1 responses and cross present antigen to CD8 T cells (Cerovic et al., 2013; 2015). Our own data also support these findings and show that all DC subsets can induce IFN-γ responses after *in vitro* incubation with SEA but we can now add that CD11b-expressing DCs are furthermore specialized to induce Th2 responses.

The fact that the transfer of SEA-loaded DCs was sufficient to induce antigen-specific Th2 responses raised the question of how DCs directly induce these responses. Many studies and our own findings have shown that Th2 responses require IL-4 to be established and maintained and IL-4 producing cells like basophils or type 2 innate lymphoid cells have been implicated in assisting Th2 differentiation (Yoshimoto, 2010; Anyan et al., 2013; Oliphant et al., 2014). However, these cells mostly reside in intestinal tissues and not in the MLNs, and our data indicate that DC-intrinsic molecules produced by the transferred DCs were sufficient to drive effective Th2 responses after transfer in the MLN. We therefore addressed whether DCs themselves provided the initial IL-4 needed for Th2 differentiation. We observed that SEA-loaded DC subsets from IL-4-deficient mice induced similar Th2 responses compared to C57BL/6 DCs after transfer, which showed that IL-4 production by DCs was not required (Fig. 6-5; 6-6), which correlated with published findings using $IL\text{-}4^{-/-}$ BMDCs (MacDonald et al., 2002).

However, it has been shown that a small number of T cells can differentiate into Th2 cells independently of IL-4 signalling, which then produce the necessary Th2 cytokines to drive an efficient immune response (Noben-Trauth et al., 2000; Jankovic et al., 2000). This could represent a possible mechanism by which Th2 responses are induced without requiring exogenous IL-4 for their initial differentiation, which could explain why the transfer of DCs in the MLN is sufficient to drive Th2 responses in our system. However, other factors, such as the production of IL-4 by other cells in the lymph node, delivery of IL-4 from the lamina propria via the lymph or the presence of memory Th2 cells, which become activated by the transferred DCs could also be involved.

Another important question was addressed by the transfer of SEA-loaded $IRF4^{f/f}$ CD11c-cre-positive DC subsets (Fig. 6-7). We had observed that $IRF4^{f/f}$ CD11c-cre-positive chimeras were impaired in inducing Th2 responses against intestinally injected *S. mansoni* eggs. Analysis of DC subsets revealed that despite the fact

that DP DCs were reduced in the small intestine, antigen uptake was not affected. In contrast, $CD11b^+$ and DP DCs were greatly reduced in the MLNs resulting in limited antigen availability and we suggested that this affected efficient Th2 priming. This corresponded with our observation that $CD11b^+$ and DP DCs are specialized for the induction of Th2 responses and the absence of these cells would likely lead to an impaired Th2 induction. However, IRF4 may also affect cell intrinsic properties in DCs that are required to induce Th2 responses, as suggested by Williams *et al.* (Williams et al., 2013). Similar effects have been shown in other cell types such as T cells, that fail to develop into Th2 cells upon IRF4 deletion (Lohoff et al., 2002), or macrophages, which depend on IRF4 to develop into alternative activated macrophages during type 2 immune responses (Satoh et al., 2010; Negishi et al., 2005). We therefore transferred SEA-loaded $IRF4^{f/f}$ CD11c-cre-positive DC subsets from the small intestinal lamina propria into wild-type recipient mice and observed that they could efficiently induce Th2 responses. DP DCs were again the most efficient DC subset to induce Th2 responses, suggesting that the deletion of IRF4 in DCs, despite causing developmental defects in DP DCs in the small intestine, did not inhibit antigen presentation and Th2 priming of these cells. These findings corresponded with our previous experiments, which showed that the presentation of OVA to OT-II cells was not inhibited in IRF4-deficient DCs and indicated that $IRF4^{f/f}$ CD11c-cre-positive DC subsets remained specialized to induce Th2 responses. This suggested that IRF4 deletion did not affect the priming capabilities of $CD11b^+$ and DP DCs but rather their migration, which limited cell and antigen availability in the MLNs and led to inefficient Th2 responses.

Having identified that SEA-loaded $CD11b^+$ and DP lymph DCs are specialized to induce Th2 responses in the intestine we addressed if the same subsets were involved under physiological conditions of egg injections. We therefore sorted lymph DC subsets directly from egg-injected donor mice and transferred the individual subsets into recipient animals. We observed that under these conditions DP DCs were solely responsible to present small intestinal egg antigen to naive T cells and induce antigen specific Th2 responses after transfer (Fig. 6-8). We suggest that this is due to the fact that lymph DP DCs are the most numerous subset to carry egg antigen from the small intestine, which we had demonstrated by the injection of fluorescently labelled SEA. It is therefore likely that under these physiological conditions the other DC subsets did not carry sufficient amounts of

antigen to induce T cell responses upon transfer, in contrast to previous experiments where all subsets were loaded with SEA *in vitro*. To clarify whether the physiological uptake of antigen or cell intrinsic properties of antigen processing and presentation predisposed DP DCs from the small intestine to be the only subset to induce antigen-specific immune responses after transfer, we incubated all DC subsets with SEA *in vitro.* As we had observed that DP DCs from the small intestine but $CD11b^+$ DCs from the colon transported the majority of egg antigen to the draining lymph nodes we aimed to directly compare these tissue-specific DCs and their capacity to induce Th2 responses. Unfortunately, we could not collect enough cells from the lymph of cMLNx animals to perform direct cell transfer experiments from colon injected donor mice. Instead, DCs were isolated from the intestinal lamina propria. Similar to our observations from SEA-loaded lymph DCs, SEA-loaded DP DCs from the small intestine were again the most efficient subset to induce Th2 responses in recipient animals. SEA-loaded $CD11b^+$ DCs from the small intestine induced could induce very low levels of Th2 responses in the MLNs of recipient mice after restimulation, whereas SEA-loaded DN and $CD103^+$ DCs could induce IFN-γ, but no Th2, responses upon transfer (Fig. 6-9). These data suggested that DP DCs from the small intestine were inherently better at either processing and presenting egg antigens or priming Th2 cells than the other DC subsets found in the small intestine. Together with our previous findings that they were the most numerous subset to transport egg antigen from the small intestine to the draining lymph nodes, and that they are the sole DC subset to induce egg antigen specific immune responses under physiological conditions, we argue that DP DCs are the primary DC subset to induce Th2 responses in the small intestine.

In contrast to small intestinal DC subsets, SEA-loaded $CD11b^+$ DCs from the colon induced the strongest Th2 responses in the recipient MLNs compared to the other DC subsets. But IFN-γ responses, similar to our previous observations, could be induced by all colonic DC subsets (Fig. 6-10). We had observed similar tissue-specific differences in previous experiments, where we observed that $CD11b^+$ DCs, and not DP DCs as for the small intestine, exclusively transported parasite antigen from the colon to the cMLNs. Furthermore, $CD11b^+$ DCs were present in increased frequency in lymph from colon injected animals, whereas DP DCs were more frequent after small intestinal injections. We therefore suggest that $CD11b^+$ DCs are the main subset to induce Th2 responses in the colon.

This is a surprising and important finding that raises many questions and could also have implications for other antigens and immune responses in these tissues, which will be discussed in more detail in chapter 7. Importantly, it remains to be determined whether both tissue-specific subsets process and present egg antigen to the same extend. It has been suggested by other studies that despite fulfilling similar functions such as oral/colonic tolerance the molecular mechanisms of these DCs are unique (Veenbergen et al., 2016), which would be interesting to address in the context of Th2 responses. Thus, this work is, to our knowledge, the first identification of the DC subsets that promote Th2 responses in the intestine, as well as the first demonstration that different DC populations from the small intestine and colon are specialized to induce these responses.

6.3 Conclusions

By transferring immune cells under the capsule of recipient mice, we were able to assess the Th2-priming potential of lymph migrating cells *in vivo*. We showed that dendritic cells, but not B cells, were responsible for priming egg antigen specific T cell responses in the MLNs, despite our findings that both cell populations carried egg antigen in lymph. T cell priming was directly mediated by the injected DCs in a MHCII-dependent manner. Dendritic cells were also able to present *in vitro* loaded SEA after transfer. Hereby, dendritic cells harvested from the small intestinal lamina propria and the lymph were able to induce IFN-γ, IL-4, IL-5 and IL-13 secreting immune responses, whereas fully mature migratory DCs from the MLNs could no longer present *in vitro* loaded SEA after transfer. We further discovered that $CD11b^+$ and DP DCs were specialized to induce Th2 responses after transfer, whereas DN and $CD103^+$ DCs only induced antigen-specific IFN-γ responses. Intracellular staining revealed that the transferred DCs induced IFN-γ expression and IL-4 and IL-13 production by separate populations of activated $CD44^{hi}$ CD4 T cells.

Immune responses could also be initiated by transferred DCs, which were unable to produce IL-4, suggesting that exogenous IL-4 was not required for the induction of Th2 responses during DC transfer into the MLNs. Furthermore, SEA-loaded DCs from in $IRF4^{f/f}$ CD11c-cre-positive mice retained their potential to prime Th2 responses when transferred into the MLNs, suggesting that limited migration of IRF4-deficient DCs, and not functional defects, impaired Th2 responses in these mice. Importantly, detailed comparisons between small intestinal and colonic DC subsets revealed that DP DCs from the small intestine were specialized and responsible to induce Th2 responses after transfer, whereas $CD11b^+$ DCs from the colon were the most efficient subset to induce Th2 responses after transfer. Thus, we have not only identified that DCs are responsible for priming Th2 cells against intestinal *S. mansoni* eggs, but that specialized intestinal DC subsets are involved. We also discovered that different cellular mechanisms exist in the small intestine and colon, revealing a hitherto unappreciated functional heterogeneity among intestinal DCs.

Chapter 7: Final Discussion

In this thesis we have identified that conventional dendritic cells are sufficient for driving Th2 responses in the intestine. As a Th2 inducing stimuli, we have used *Schistosoma mansoni* eggs, which drive potent type 2 immune responses and cause damage and induce granuloma formation, which lead to organ fibrosis and morbidity during chronic infection. We have furthermore revealed the mechanisms by which the induction of these Th2 responses is dependent on the transcription factor IRF4, and that different tissue-specific subsets of dendritic cells are specialized to drive Th2 responses in the small intestine and colon. In this final chapter an overview of the novel insights into DC functionality regarding Th2 responses will be described and the immunological relevance of these findings will be discussed.

We have developed an experimental immunization protocol that for the first time allows the detailed assessment of the induction of Th2 responses in the intestine. The delivery of eggs into the intestinal subserosa allowed us to control parameters such as the amount and location of deposited eggs and the timing of the induction period, which are impossible to define during live infection. The definition of these parameters is crucial to investigate Th2 cell induction *in vivo* and separate it from other immunological events like Th2 cell maintenance or restimulation. Furthermore, the importance of understanding the induction of Th2 responses in the intestine is highlighted by the fact that the intestine is heavily affected by penetrating and trapped eggs during live *S. mansoni* infection and is the target of numerous helminths parasite infections (Grencis, 2015). During live infections, *S. mansoni* eggs normally penetrate through the intestinal wall to be shed in the faeces to continue the parasite's life cycle. However, not all eggs succeed in penetrating the intestine and many remain trapped within the tissue, where they are encased by granulomas and lead to tissue fibrosis (Pearce and MacDonald, 2002). Our egg injection technique is specifically designed to enable the delivery of eggs into their physiological location and represent trapped eggs within the tissue *in vivo*, which is a distinct refinement over previously used approaches. We detected strong antigen specific immune responses in the draining mesenteric lymph nodes that were comprised of IFN-γ and Th2 responses, which are typically mounted against *S. mansoni* eggs (MacDonald and Pearce, 2002; MacDonald and Maizels, 2008; Cook et al., 2015). Thus, the experimental immunization of *S.*

mansoni eggs enabled us to focus on and precisely examine the physiologically relevant mechanisms of Th2 induction in the intestine with unprecedented clarity *in vivo*.

It has been demonstrated that the transcription factor IRF4 is an important regulator of type 2 immunity and controls the development of Th2 cells, post-germinal center plasma cells, alternatively activated macrophages and CD11b-expressing dendritic cells. We focused on the effects of IRF4 deficiency on dendritic cells and discovered that Th2 responses against *S. mansoni* eggs were impaired in IRF4$^{f/f}$ CD11c-cre mice, which was accompanied by an increased IFN-γ response. It had been previously observed that type 2 immune responses are abrogated during allergen challenge in the lung (Williams et al., 2013), but this is the first demonstration that IRF4 is also required by DCs to drive Th2 responses against parasite antigens. IRF4 is not only involved in the development of DCs but has also been shown to play a role in dendritic cell migration and directly regulating BMDC-driven Th2 differentiation via peptide-MHC class II complex formation (Vander Lugt et al., 2014) and the expression of IL-10, IL-33 and OX40L (Williams et al., 2013). In the intestine we verified that IRF4 affected intestinal DC subset development and diminished the number of CD11b-expressing DCs in the MLNs, as previously described (Persson et al., 2013b). Importantly, we were able to reveal a direct functional importance, as this dramatic decrease of CD11b-expressing DCs in the MLNs led to insufficient transport of parasite antigen to the lymph nodes. We suggest that the decrease of CD11b-expressing DCs and limited amounts of antigen are key factors that impair the induction of Th2 responses in IRF4$^{f/f}$ CD11c-cre mice. In contrast to published findings, we found that IRF4 deficient DCs from the intestine could prime naive T cells and induce Th2 responses when transferred into recipient mice, suggesting that defective migration, rather than molecular changes that impaired T cell priming and differentiation were involved. Further experiments, like the assessment of lymph migrating DC subsets from IRF4$^{f/f}$ CD11c-cre mice could clarify if defects in cell migration or cell survival lead to the diminished number of CD11b-expressing DCs in the MLNs.

As determined by our experiments in IRF4$^{f/f}$ CD11c-cre mice and shown in previous studies using CD11c-DTR mice (Phythian-Adams et al., 2010), CD11c^{+} cells are necessary for the establishment of Th2 responses against *S. mansoni*

egg antigens. However, it was unknown whether DCs are also sufficient to drive effective Th2 responses. We have used innovative approaches to isolate DCs from the exact tissues involved in Th2 responses to *S. mansoni* eggs, be it the intestinal lamina propria, lymph or individual small intestinal or colonic draining mesenteric lymph nodes (MLNs). To identify DCs populations sufficient to prime intestinal Th2 responses we developed techniques for transferring purified DCs into recipient animals. DCs were delivered under the capsule of the MLN, precisely where they would enter the lymph node under normal physiological conditions. By efficiently delivering the appropriate DCs into the correct location, we could directly and precisely examine their Th2-inducing functions. This painstaking approach to the use of precise tissue-derived DCs was critical, as we were thus able to discover that immature intestinal DCs, as well as semi-mature lymph migrating DCs were sufficient to drive Th2 responses against *S. mansoni* egg antigens, whereas fully mature MLN DCs no longer presented exogenously provided egg antigens. This evidence demonstrates for the first time that intestinal DCs are sufficient to drive Th2 responses (Fig. 7-1).

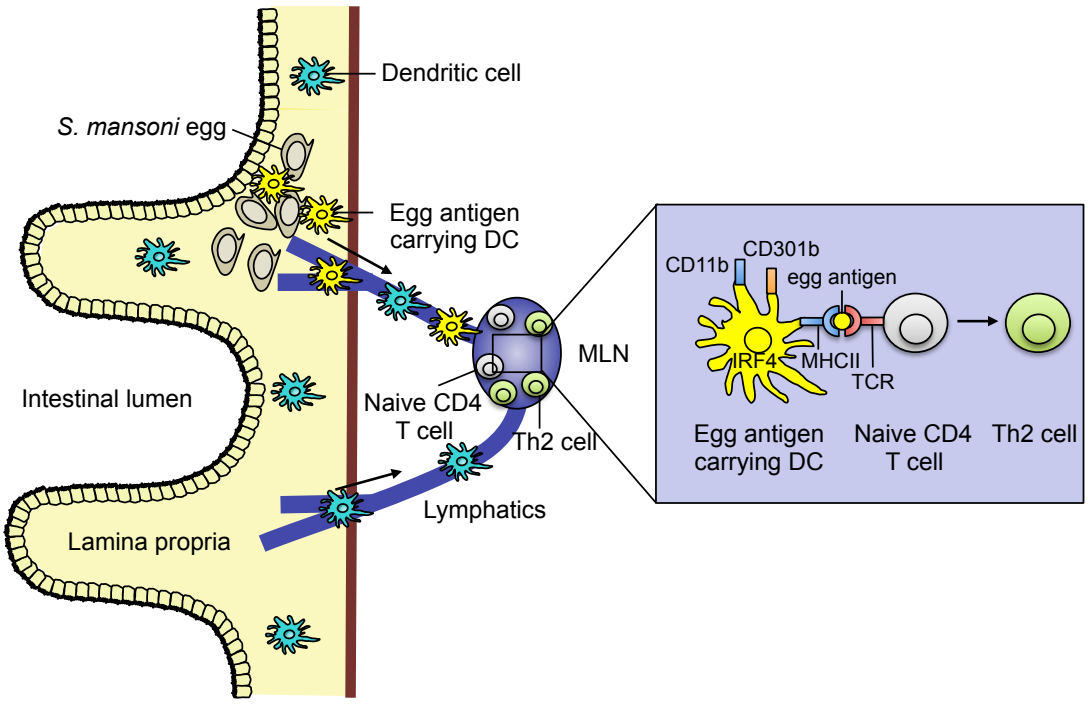

Figure 7-1. Induction of Th2 responses against *Schistosoma mansoni* eggs in the intestine

In our experimental immunization model *S. mansoni* eggs are injected into the intestinal subserosa. Egg antigen is taken up by DCs, which migrate to the draining mesenteric lymph nodes (MLNs). We have identified that these DCs express CD11b, CD301b and IRF4 and in the small intestine the subset of $CD11b^{+}CD103^{+}$ DP DCs and in the colon $CD11b^{+}CD103^{-}$ DCs are predominantly involved in this process. Within the MLN these DCs present egg antigen to naive T cells via MHCII and drive their differentiation into antigen specific Th2 cells. $CD11b^{+}CD103^{+}$ DP DCs from the small intestine and $CD11b^{+}CD103^{-}$ DCs from the colon are specialized to drive this differentiation and are necessary and sufficient to prime Th2 cells against *S. mansoni* egg antigens in the intestine.

Other models have suggested that apart from DCs other cells, like basophils or ILC2s, are required to provide signals necessary for the full differentiation of Th2 cells. However, in our transfer system DCs were sufficient to drive competent immune responses that were comparable to the injections of eggs into the intestine. Thus, conventional intestinal DCs are fully capable of inducing Th2 responses against *S. mansoni* egg antigens upon transfer, without the explicit requirement for other accessory cells that also required transfer. In an attempt to define the molecular mechanisms that could be involved, we determined that the incubation with soluble egg antigens (SEA) had limited effects on the expression of costimulatory molecules and that the secretion of IL-4 by DCs was not required to drive Th2 responses, which was in accordance to published findings (MacDonald et al., 2002). However, we were not able to find a distinctive molecular signature, and the molecular mechanisms by which intestinal DCs induce Th2 differentiation remain elusive. Technologies, like RNA sequencing,

could reveal changes in gene expression after incubation with *S. mansoni* and may thus suggest novel targets and pathways that might be involved.

Recent work in DC biology has demonstrated that specific DC subsets are specialized to perform different roles in T helper cell differentiation. This has been demonstrated for Th1, Th17 and Treg cells in the intestines but it is unclear if it also applies to the induction of Th2 cells. Using our state of the art techniques of intestinal DC transfers we were not only able to determine that intestinal DCs were sufficient to drive Th2 responses against *S. mansoni* egg antigens, but also investigate if specific DC subsets were required. Our findings from IRF4$^{f/f}$ CD11c-cre mice suggested that CD11b-expressing DCs are involved; as these are greatly reduced in these animals, whereas CD103^{+} DCs were not affected. In the intestine two DC subsets exist that express CD11b: CD11b^{+}CD103^{-} (CD11b^{+}) DCs, and CD11b^{+}CD103^{+} double positive (DP) DCs. The composition of these subsets differs between the small intestine and the colon and DP DCs are the predominant subset in the small intestine, whereas CD11b^{+} DCs are more abundant in the colon (Denning et al., 2011). To selectively observe if the tissue specific composition of DC subsets also translated into functional differences we took special care to selectively isolating them. The separate collection of the small intestine and the colon, the selective removal of the sMLNs or cMLNs in lymphadenectomized mice, which allowed the collection of small intestinal or colon migrating DCs in lymph, and the individual harvesting of sMLNs and cMLNs ensured that tissue-specific DCs were collected.

We observed that after egg injection in the small intestine, DP DCs migrated in increased frequency in lymph, whereas after colonic egg injection CD11b^{+} DCs were increased in lymph. Furthermore, these specific subsets were also responsible for carrying fluorescently-labelled SEA from the lamina propria of the small intestine or colon to the draining lymph nodes. This suggested that DP DCs in the small intestine and CD11b^{+} DCs in the colon respond to egg antigens and are specialized to carry them to the draining lymph nodes. Using our cell transfer system we were able to demonstrate that these tissue-specific DC subsets were furthermore responsible for priming Th2 cells (Fig. 7-1). We have thus demonstrated that specific subsets of intestinal DCs are specialized to prime Th2 cells. We speculate that this capacity extends beyond antigens from *S. mansoni* eggs but might also be relevant for other parasitic antigens or allergens. Many

different food allergies exist and specific food allergens could be used to determine if the same DC subsets are involved in the induction of allergic Th2 responses in the intestine. Identifying and targeting these specific subsets could be beneficial for the modulation of pathological Th2 responses, including chronic schistosomiasis, and an array of allergic diseases affecting the intestine.

We believe that our discovery that distinct subsets of DCs prime Th2 responses in the small intestine and colon represents a paradigm-shifting finding and is also relevant for intestinal immunology beyond type 2 immunity.

We demonstrate that distinct DC subsets within different tissues can carry out the same functions. Thus, tissue-specific factors acting on DC precursors can drive independent patterning of phenotypic and functional DC characteristics. This not only highlights the need for accurate markers of DCs' functional properties, but also brings new complexity to our understanding of the tissue-specific cues that influence DC differentiation, in the intestine and elsewhere. Thus, tissue-specific factors that induce the expression of particular subset-specific surface markers can no longer be assumed to generate the same functional characteristics. The differences in the functions of DC subsets between the small intestine and colon also provide clear evidence that the induction of immune responses in these tissues is distinct. This idea is supported by recent findings that have demonstrated that oral tolerance in the small intestine and colonic tolerance are driven by distinct subsets of tissue-specific DCs (Veenbergen et al., 2016). Beyond advancing our understanding of the immunological differences between these tissues this raises the possibility that diseases in the small intestine and colon could also be influenced by distinct subsets of DCs. Many important infections and diseases of the intestine, (e.g. parasite infections, Crohn's disease, ulcerative colitis and celiac disease), selectively affect the small intestine or colon and dendritic cells have been shown to contribute to disease induction or progression. Identifying the role of specific DC subsets could reveal novel approaches that could be precisely and independently targeted to interfere with these diseases. Thus, our identification of the tissue-specific DC subsets that induce Th2 responses against *Schistosoma mansoni* eggs in the intestine reveals novel details for intestinal type 2 immune responses, and also impacts our understanding of intestinal immune responses in general, by demonstrating that different tissue-specific subsets are involved.

7.1 Conclusions

Here we have developed an immunization protocol that allows for the physiological delivery of *Schistosoma mansoni* eggs into the intestine to study the induction of Th2 responses induced by egg antigens. We have revealed that conventional DCs are responsible for carrying egg antigen to the draining mesenteric lymph nodes, where they prime naive T cells and induce antigen specific IFN-γ and Th2 responses. In fact, intestinal IRF4 and CD11b-expressing DCs are specialized to drive Th2 responses and distinct populations play a role in the small intestine and colon, whereas antigen specific IFN-γ can be induced by all DC populations. Thus, we have identified the specific DC subsets that drive intestinal Th2 responses and revealed a hitherto unappreciated functional heterogeneity among intestinal DCs between the small intestine and colon. These findings not only advance our knowledge of intestinal Th2 responses against parasite antigens but also impact our immunological understanding of the small intestine and colon. Future experiments will demonstrate if these DC subsets also drive Th2 responses against other intestinal parasites or allergic conditions and if distinct subsets of CD11b-expressing DCs from the small intestine or colon are involved in driving other immunological conditions like Crohn's disease, ulcerative colitis and celiac disease.

Chapter 8: References

Alves, C.C., N. Araujo, G.D. Cassali, and C.T. Fonseca. 2016. Parasitological, Pathological, and Immunological Parameters Associated with Schistosoma mansoni Infection and Reinfection in BALB/c AND C57BL/6 Mice. *J. Parasitol.* 102:336–341.

Amsen, D., J.M. Blander, G.R. Lee, K. Tanigaki, T. Honjo, and R.A. Flavell. 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell*. 117:515–526.

Antignano, F., S.C. Mullaly, K. Burrows, and C. Zaph. 2011. Trichuris muris infection: a model of type 2 immunity and inflammation in the gut. *J Vis Exp*. 51.

Anyan, W.K., T. Seki, T. Kumagai, K. Obata-Ninomiya, R. Furushima-Shimogawara, B. Kwansa-Bentum, N. Akao, K.M. Bosompem, D.A. Boakye, M.D. Wilson, H. Karasuyama, and N. Ohta. 2013. Basophil depletion downregulates Schistosoma mansoni egg-induced granuloma formation. *Parasitol Int*. 62:508–513.

Arima, K., N. Watanabe, S. Hanabuchi, M. Chang, S.-C. Sun, and Y.-J. Liu. 2010. Distinct signal codes generate dendritic cell functional plasticity. *Sci Signal*. 3.

Bain, C.C., and A.M. Mowat. 2014. Macrophages in intestinal homeostasis and inflammation. *Immunol Rev*. 260:102–117.

Bain, C.C., C.L. Scott, H. Uronen-Hansson, S. Gudjonsson, O. Jansson, O. Grip, M. Guilliams, B. Malissen, W.W. Agace, and A.M. Mowat. 2013. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol*. 6:498–510.

Bajaña, S., K. Roach, S. Turner, J. Paul, and S. Kovats. 2012. IRF4 promotes cutaneous dendritic cell migration to lymph nodes during homeostasis and inflammation. *J Immunol*. 189:3368–3377.

Bekiaris, V., E.K. Persson, and W.W. Agace. 2014. Intestinal dendritic cells in the regulation of mucosal immunity. *Immunol Rev*. 260:86–101.

Bell, B.D., M. Kitajima, R.P. Larson, T.A. Stoklasek, K. Dang, K. Sakamoto, K.-U. Wagner, D.H. Kaplan, B. Reizis, L. Hennighausen, and S.F. Ziegler. 2013. The transcription factor STAT5 is critical in dendritic cells for the development of TH2 but not TH1 responses. *Nat Immunol*. 14:364–371.

Bogunovic, M., F. Ginhoux, J. Helft, L. Shang, D. Hashimoto, M. Greter, K. Liu, C. Jakubzick, M.A. Ingersoll, M. Leboeuf, E.R. Stanley, M. Nussenzweig, S.A. Lira, G.J. Randolph, and M. Merad. 2009. Origin of the lamina propria dendritic cell network. *Immunity*. 31:513–525.

Braun, A., T. Worbs, G.L. Moschovakis, S. Halle, K. Hoffmann, J. Bölter, A. Münk, and R. Förster. 2011. Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration. *Nat Immunol*. 12:879–887.

Brinkmann, V., J.G. Cyster, and T. Hla. 2004. FTY720: sphingosine 1-phosphate receptor-1 in the control of lymphocyte egress and endothelial barrier function. *Am J Transplant*. 6:1019–1025.

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

Campbell, D.J., and E.C. Butcher. 2002. Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues. *J Exp Med*. 195:135–141.

Carter, P.B., and F.M. Collins. 1974. The route of enteric infection in normal mice. *J Exp Med*. 139:1189–1203.

Cerovic, V., C.C. Bain, A.M. Mowat, and S.W.F. Milling. 2014. Intestinal macrophages and dendritic cells: what's the difference? *Trends Immunol*. 35:270–277.

Cerovic, V., C.D. Jenkins, A.G.C. Barnes, S.W.F. Milling, G.G. MacPherson, and L.S. Klavinskis. 2009. Hyporesponsiveness of intestinal dendritic cells to TLR stimulation is limited to TLR4. *J Immunol*. 182:2405–2415.

Cerovic, V., S.A. Houston, C.L. Scott, A. Aumeunier, U. Yrlid, A.M. Mowat, and S.W.F. Milling. 2013. Intestinal CD103(-) dendritic cells migrate in lymph and prime effector T cells. *Mucosal Immunol*. 6:104–113.

Cerovic, V., S.A. Houston, J. Westlund, L. Utriainen, E.S. Davison, C.L. Scott, C.C. Bain, T. Joeris, W.W. Agace, R.A. Kroczek, A.M. Mowat, U. Yrlid, and S.W.F. Milling. 2015. Lymph-borne CD8α+ dendritic cells are uniquely able to cross-prime CD8+ T cells with antigen acquired from intestinal epithelial cells. *Mucosal Immunol*. 8:38–48.

Cervi, L., A.S. MacDonald, C. Kane, F. Dzierszinski, and E.J. Pearce. 2004. Cutting edge: dendritic cells copulsed with microbial and helminth antigens undergo modified maturation, segregate the antigens to distinct intracellular compartments, and concurrently induce microbe-specific Th1 and helminth-specific Th2 responses. *J Immunol*. 172:2016–2020.

Chang, Y.-J., H.Y. Kim, L.A. Albacker, N. Baumgarth, A.N.J. McKenzie, D.E. Smith, R.H. DeKruyff, and D.T. Umetsu. 2011. Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol*. 12:631–638.

Cheever, A.W., J.A. Lenzi, H.L. Lenzi, and Z.A. Andrade. 2002. Experimental models of Schistosoma mansoni infection. *Mem. Inst. Oswaldo Cruz*. 97:917–940.

Chen, L., and D.B. Flies. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol*. 13:227–242.

Cheroutre, H., F. Lambolez, and D. Mucida. 2011. The light and dark sides of intestinal intraepithelial lymphocytes. *Nat Rev Immunol*. 11:445–456.

Chiaramonte, M.G., D.D. Donaldson, A.W. Cheever, and T.A. Wynn. 1999. An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-

dominated inflammatory response. *J Clin Invest*. 104:777–785.

Cliffe, L.J., and R.K. Grencis. 2004. The Trichuris muris system: a paradigm of resistance and susceptibility to intestinal nematode infection. *Adv Parasitol*. 57:255–307.

Connor, L.M., S.C. Tang, M. Camberis, G. Le Gros, and F. Ronchese. 2014. Helminth-Conditioned Dendritic Cells Prime CD4+ T Cells to IL-4 Production In Vivo. *J Immunol*. 193:2709–2717.

Constant, S.L., and K. Bottomly. 1997. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol*. 15:297–322.

Cook, P.C., H. Owen, A.E.E.M. Deaton, J.G. Borger, S.L. Brown, T. Clouaire, G.-R. Jones, L.H. Jones, R.J. Lundie, A.K. Marley, V.L. Morrison, A.T. Phythian-Adams, E. Wachter, L.M. Webb, T.E. Sutherland, G.D. Thomas, J.R. Grainger, J. Selfridge, A.N.J. McKenzie, J.E. Allen, S.C. Fagerholm, R.M. Maizels, A.C. Ivens, A. Bird, and A.S. MacDonald. 2015. A dominant role for the methyl-CpG-binding protein Mbd2 in controlling Th2 induction by dendritic cells. *Nat Commun*. 6:1–11.

Coombes, J.L., K.R.R. Siddiqui, C.V. Arancibia-Cárcamo, J. Hall, C.-M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med*. 204:1757–1764.

de Jesus, A.R., A. Silva, L.B. Santana, A. Magalhães, A.A. de Jesus, R.P. de Almeida, M.A.V. Rêgo, M.N. Burattini, E.J. Pearce, and E.M. Carvalho. 2002. Clinical and immunologic evaluation of 31 patients with acute schistosomiasis mansoni. *J Infect Dis*. 185:98–105.

de Jong, E.C., P.L. Vieira, P. Kalinski, J.H.N. Schuitemaker, Y. Tanaka, E.A. Wierenga, M. Yazdanbakhsh, and M.L. Kapsenberg. 2002. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals. *J Immunol*. 168:1704–1709.

De Smedt, T., M. Van Mechelen, G. De Becker, J. Urbain, O. Leo, and M. Moser. 1997. Effect of interleukin-10 on dendritic cell maturation and function. *Eur J Immunol*. 27:1229–1235.

Denning, T.L., B.A. Norris, O. Medina-Contreras, S. Manicassamy, D. Geem, R. Madan, C.L. Karp, and B. Pulendran. 2011. Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. *J Immunol*. 187:733–747.

Dunne, D.W., A.E. Butterworth, A.J. Fulford, H.C. Kariuki, J.G. Langley, J.H. Ouma, A. Capron, R.J. Pierce, and R.F. Sturrock. 1992. Immunity after treatment of human schistosomiasis: association between IgE antibodies to adult worm antigens and resistance to reinfection. *Eur J Immunol*. 22:1483–1494.

Dunne, D.W., and A. Cooke. 2005. A worm's eye view of the immune system: consequences for evolution of human autoimmune disease. *Nat Rev Immunol*. 5:420–426.

Dunne, D.W., and E.J. Pearce. 1999. Immunology of hepatosplenic

schistosomiasis mansoni: a human perspective. *Microbes Infect*. 1:553–560.

Dunne, D.W., and M.J. Doenhoff. 1983. Schistosoma mansoni egg antigens and hepatocyte damage in infected T cell-deprived mice. *Contrib Microbiol Immunol*. 7:22–29.

Edelson, B.T., W. KC, R. Juang, M. Kohyama, L.A. Benoit, P.A. Klekotka, C. Moon, J.O.R.C. Albring, W. Ise, D.G. Michael, D. Bhattacharya, T.S. Stappenbeck, M.J. Holtzman, S.-S.J. Sung, T.L. Murphy, K. Hildner, and K.M. Murphy. 2010. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. *J Exp Med*. 207:823–836.

Everts, B., G. Perona-Wright, H.H. Smits, C.H. Hokke, A.J. van der Ham, C.M. Fitzsimmons, M.J. Doenhoff, J.U.R. van der Bosch, K. Mohrs, H. Haas, M. Mohrs, M. Yazdanbakhsh, and G. Schramm. 2009. Omega-1, a glycoprotein secreted by Schistosoma mansoni eggs, drives Th2 responses. *J Exp Med*. 206:1673–1680.

Fallon, P.G., E.J. Richardson, G.J. McKenzie, and A.N. McKenzie. 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*. 164:2585–2591.

Fallon, P.G., S.J. Ballantyne, N.E. Mangan, J.L. Barlow, A. Dasvarma, D.R. Hewett, A. McIlgorm, H.E. Jolin, and A.N.J. McKenzie. 2006. Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J Exp Med*. 203:1105–1116.

Farache, J., I. Koren, I. Milo, I. Gurevich, K.-W. Kim, E. Zigmond, G.C. Furtado, S.A. Lira, and G. Shakhar. 2013. Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity*. 38:581–595.

Flores-Langarica, A., J.L. Marshall, J. Hitchcock, C. Cook, J. Jobanputra, S. Bobat, E.A. Ross, R.E. Coughlan, I.R. Henderson, S. Uematsu, S. Akira, and A.F. Cunningham. 2012. Systemic flagellin immunization stimulates mucosal CD103+ dendritic cells and drives Foxp3+ regulatory T cell and IgA responses in the mesenteric lymph node. *J Immunol*. 189:5745–5754.

Flynn, S., K.M. Toellner, C. Raykundalia, M. Goodall, and P. Lane. 1998. CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blr-1. *J Exp Med*. 188:297–304.

Fogg, D.K., C. Sibon, C. Miled, S. Jung, P. Aucouturier, D.R. Littman, A. Cumano, and F. Geissmann. 2006. A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science*. 311:83–87.

Förster, R., A.C. Davalos-Misslitz, and A. Rot. 2008. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol*. 8:362–371.

Fujimoto, K., T. Karuppuchamy, N. Takemura, M. Shimohigoshi, T. Machida, Y. Haseda, T. Aoshi, K.J. Ishii, S. Akira, and S. Uematsu. 2011. A new subset of CD103+CD8alpha+ dendritic cells in the small intestine expresses TLR3, TLR7, and TLR9 and induces Th1 response and CTL activity. *J Immunol*. 186:6287–6295.

Gao, Y., S.A. Nish, R. Jiang, L. Hou, P. Licona-Limón, J.S. Weinstein, H. Zhao, and R. Medzhitov. 2013. Control of T Helper 2 Responses by Transcription Factor IRF4-Dependent Dendritic Cells. *Immunity*. 39:722–732.

Gautier, E.L., T. Shay, J. Miller, M. Greter, C. Jakubzick, S. Ivanov, J. Helft, A. Chow, K.G. Elpek, S. Gordonov, A.R. Mazloom, A. Ma'ayan, W.-J. Chua, T.H. Hansen, S.J. Turley, M. Merad, G.J. Randolph, Immunological Genome Consortium. 2012. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol*. 13:1118–1128.

Gerbe, F., E. Sidot, D.J. Smyth, M. Ohmoto, I. Matsumoto, V. Dardalhon, P. Cesses, L. Garnier, M. Pouzolles, B. Brulin, M. Bruschi, Y. Harcus, V.S. Zimmermann, N. Taylor, R.M. Maizels, and P. Jay. 2016. Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites. *Nature*. 529:226–230.

Geuking, M.B., J. Cahenzli, M.A.E. Lawson, D.C.K. Ng, E. Slack, S. Hapfelmeier, K.D. McCoy, and A.J. Macpherson. 2011. Intestinal bacterial colonization induces mutualistic regulatory T cell responses. *Immunity*. 34:794–806.

Gordon, S. 2003. Alternative activation of macrophages. *Nat Rev Immunol*. 3:23–35.

Grencis, R.K. 2015. Immunity to helminths: resistance, regulation, and susceptibility to gastrointestinal nematodes. *Annu Rev Immunol*. 33:201–225.

Grencis, R.K., N.E. Humphreys, and A.J. Bancroft. 2014. Immunity to gastrointestinal nematodes: mechanisms and myths. *Immunol Rev*. 260:183–205.

Guilliams, M., F. Ginhoux, C. Jakubzick, S.H. Naik, N. Onai, B.U. Schraml, E. Segura, R. Tussiwand, and S. Yona. 2014. Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol*. 14:571–578.

Habtezion, A., L.P. Nguyen, H. Hadeiba, and E.C. Butcher. 2016. Leukocyte Trafficking to the Small Intestine and Colon. *Gastroenterology*. 150:340–354.

Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Müller, T. Sparwasser, R. Förster, and O. Pabst. 2011. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity*. 34:237–246.

Hase, K., K. Kawano, T. Nochi, G.S. Pontes, S. Fukuda, M. Ebisawa, K. Kadokura, T. Tobe, Y. Fujimura, S. Kawano, A. Yabashi, S. Waguri, G. Nakato, S. Kimura, T. Murakami, M. Iimura, K. Hamura, S.-I. Fukuoka, A.W. Lowe, K. Itoh, H. Kiyono, and H. Ohno. 2009. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. *Nature*. 462:226–230.

Helft, J., J. Böttcher, P. Chakravarty, S. Zelenay, J. Huotari, B.U. Schraml, D. Goubau, and C. Reis e Sousa. 2015. GM-CSF Mouse Bone Marrow Cultures Comprise a Heterogeneous Population of CD11c(+)MHCII(+) Macrophages and Dendritic Cells. *Immunity*. 42:1197–1211.

Hesse, M., C.A. Piccirillo, Y. Belkaid, J. Prufer, M. Mentink-Kane, M. Leusink, A.W. Cheever, E.M. Shevach, and T.A. Wynn. 2004. The pathogenesis of

schistosomiasis is controlled by cooperating IL-10-producing innate effector and regulatory T cells. *J Immunol*. 172:3157–3166.

Hildner, K., B.T. Edelson, W.E. Purtha, M. Diamond, H. Matsushita, M. Kohyama, B. Calderon, B.U. Schraml, E.R. Unanue, M.S. Diamond, R.D. Schreiber, T.L. Murphy, and K.M. Murphy. 2008. Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. *Science*. 322:1097–1100.

Hoffmann, K.F., A.W. Cheever, and T.A. Wynn. 2000. IL-10 and the dangers of immune polarization: excessive type 1 and type 2 cytokine responses induce distinct forms of lethal immunopathology in murine schistosomiasis. *J Immunol*. 164:6406–6416.

Hotez, P.J., P.J. Brindley, J.M. Bethony, C.H. King, E.J. Pearce, and J. Jacobson. 2008. Helminth infections: the great neglected tropical diseases. *J Clin Invest*. 118:1311–1321.

Houston, S.A., V. Cerovic, C. Thomson, J. Brewer, A.M. Mowat, and S. Milling. 2016. The lymph nodes draining the small intestine and colon are anatomically separate and immunologically distinct. *Mucosal Immunol*. 9:468–478.

Howitt, M.R., S. Lavoie, M. Michaud, A.M. Blum, S.V. Tran, J.V. Weinstock, C.A. Gallini, K. Redding, R.F. Margolskee, L.C. Osborne, D. Artis, and W.S. Garrett. 2016. Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut. *Science*. 351:1329–1333.

Hsieh, C.S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science*. 260:547–549.

Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992a. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med*. 176:1693–1702.

Inaba, K., R.M. Steinman, M.W. Pack, H. Aya, M. Inaba, T. Sudo, S. Wolpe, and G. Schuler. 1992b. Identification of proliferating dendritic cell precursors in mouse blood. *J Exp Med*. 175:1157–1167.

Ito, T., Y.-H. Wang, O. Duramad, T. Hori, G.J. Delespesse, N. Watanabe, F.X.-F. Qin, Z. Yao, W. Cao, and Y.-J. Liu. 2005. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med*. 202:1213–1223.

Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S.-Y. Song. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity*. 21:527–538.

Jankovic, D., M.C. Kullberg, N. Noben-Trauth, P. Caspar, W.E. Paul, and A. Sher. 2000. Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in vitro development of CD4+ lymphocytes with a Th2 cytokine profile. *J Immunol*. 164:3047–3055.

Jenkins, S.J., G. Perona-Wright, A.G.F. Worsley, N. Ishii, and A.S. MacDonald. 2007. Dendritic Cell Expression of OX40 Ligand Acts as a Costimulatory, Not

Polarizing, Signal for Optimal Th2 Priming and Memory Induction In Vivo. *J Immunol*.

Jenkins, S.J., J.P. Hewitson, G.R. Jenkins, and A.P. Mountford. 2005. Modulation of the host's immune response by schistosome larvae. *Parasite Immunol*. 27:385–393.

Joffre, O., M.A. Nolte, R. Spörri, and C. Reis e Sousa. 2009. Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. *Immunol Rev*. 227:234–247.

Joffre, O., T. Santolaria, D. Calise, T. Al Saati, D. Hudrisier, P. Romagnoli, and J.P.M. van Meerwijk. 2008. Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. *Nat Med*. 14:88–92.

Johansson-Lindbom, B., and W.W. Agace. 2007. Generation of gut-homing T cells and their localization to the small intestinal mucosa. *Immunol Rev*. 215:226–242.

Johansson-Lindbom, B., M. Svensson, O. Pabst, C. Palmqvist, G. Marquez, R. Förster, and W.W. Agace. 2005. Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing. *J Exp Med*. 202:1063–1073.

Johnston, L.K., K.B. Chien, and P.J. Bryce. 2014. The immunology of food allergy. *J Immunol*. 192:2529–2534.

Jung, C., J.-P. Hugot, and F. Barreau. 2010. Peyer's Patches: The Immune Sensors of the Intestine. *Int J Inflam*. 2010:823710.

Kaiko, G.E., J.C. Horvat, K.W. Beagley, and P.M. Hansbro. 2008. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology*. 123:326–338.

Kanaya, T., K. Hase, D. Takahashi, S. Fukuda, K. Hoshino, I. Sasaki, H. Hemmi, K.A. Knoop, N. Kumar, M. Sato, T. Katsuno, O. Yokosuka, K. Toyooka, K. Nakai, A. Sakamoto, Y. Kitahara, T. Jinnohara, S.J. McSorley, T. Kaisho, I.R. Williams, and H. Ohno. 2012. The Ets transcription factor Spi-B is essential for the differentiation of intestinal microfold cells. *Nat Immunol*. 13:729–736.

Karsunky, H., M. Merad, A. Cozzio, I.L. Weissman, and M.G. Manz. 2003. Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo. *J Exp Med*. 198:305–313.

Kim, S.V., W.V. Xiang, C. Kwak, Y. Yang, X.W. Lin, M. Ota, U. Sarpel, D.B. Rifkin, R. Xu, and D.R. Littman. 2013. GPR15-mediated homing controls immune homeostasis in the large intestine mucosa. *Science*. 340:1456–1459.

Klaver, E.J., L.M. Kuijk, T.K. Lindhorst, R.D. Cummings, and I. van Die. 2015. Schistosoma mansoni Soluble Egg Antigens Induce Expression of the Negative Regulators SOCS1 and SHP1 in Human Dendritic Cells via Interaction with the Mannose Receptor. *PloS One*. 10:e0124089.

Klein, U., S. Casola, G. Cattoretti, Q. Shen, M. Lia, T. Mo, T. Ludwig, K. Rajewsky, and R. Dalla-Favera. 2006. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nat Immunol*. 7:773–782.

Knights, D., K.G. Lassen, and R.J. Xavier. 2013. Advances in inflammatory bowel disease pathogenesis: linking host genetics and the microbiome. *Gut*. 62:1505–1510.

Kool, M., T. Soullie, M. van Nimwegen, M.A.M. Willart, F. Muskens, S. Jung, H.C. Hoogsteden, H. Hammad, and B.N. Lambrecht. 2008. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med*. 205:869–882.

Kullberg, M.C., A.G. Rothfuchs, D. Jankovic, P. Caspar, T.A. Wynn, P.L. Gorelick, A.W. Cheever, and A. Sher. 2001. Helicobacter hepaticus-induced colitis in interleukin-10-deficient mice: cytokine requirements for the induction and maintenance of intestinal inflammation. *Infect Immun*. 69:4232–4241.

Kumamoto, Y., M. Linehan, J.S. Weinstein, B.J. Laidlaw, J.E. Craft, and A. Iwasaki. 2013. CD301b+ Dermal Dendritic Cells Drive T Helper 2 Cell-Mediated Immunity. *Immunity*. 39:733–743.

Kunkel, E.J., C.H. Kim, N.H. Lazarus, M.A. Vierra, D. Soler, E.P. Bowman, and E.C. Butcher. 2003. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *J Clin Invest*. 111:1001–1010.

Kunkel, E.J., J.J. Campbell, G. Haraldsen, J. Pan, J. Boisvert, A.I. Roberts, E.C. Ebert, M.A. Vierra, S.B. Goodman, M.C. Genovese, A.J. Wardlaw, H.B. Greenberg, C.M. Parker, E.C. Butcher, D.P. Andrew, and W.W. Agace. 2000. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. *J Exp Med*. 192:761–768.

Le Gros, G., S.Z. Ben-Sasson, R. Seder, F.D. Finkelman, and W.E. Paul. 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*. 172:921–929.

Lelouard, H., M. Fallet, B. de Bovis, S. Méresse, and J.-P. Gorvel. 2012. Peyer's patch dendritic cells sample antigens by extending dendrites through M cell-specific transcellular pores. *Gastroenterology*. 142:592–601.e3.

León, B., A. Ballesteros-Tato, and F.E. Lund. 2014. Dendritic Cells and B Cells: Unexpected Partners in Th2 Development. *J Immunol*. 193:1531–1537.

Leung, S., D. Smith, A. Myc, J. Morry, and J.R. Baker. 2013. OT-II TCR transgenic mice fail to produce anti-ovalbumin antibodies upon vaccination. *Cel Immunol*. 282:79–84.

Licona-Limón, P., L.K. Kim, N.W. Palm, and R.A. Flavell. 2013. TH2, allergy and group 2 innate lymphoid cells. *Nat Immunol*. 14:536–542.

Liu, K., G.D. Victora, T.A. Schwickert, P. Guermonprez, M.M. Meredith, K. Yao, F.-F. Chu, G.J. Randolph, A.Y. Rudensky, and M. Nussenzweig. 2009. In vivo analysis of dendritic cell development and homeostasis. *Science*. 324:392–397.

Lohoff, M., H.-W. Mittrücker, S. Prechtl, S. Bischof, F. Sommer, S. Kock, D.A. Ferrick, G.S. Duncan, A. Gessner, and T.W. Mak. 2002. Dysregulated T helper

cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci U S A*. 99:11808–11812.

Mabbott, N.A., D.S. Donaldson, H. Ohno, I.R. Williams, and A. Mahajan. 2013. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunol*. 6:666–677.

Macatonia, S.E., S.C. Knight, A.J. Edwards, S. Griffiths, and P. Fryer. 1987. Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies. *J Exp Med*. 166:1654–1667.

MacDonald, A.S., A.D. Straw, B. Bauman, and E.J. Pearce. 2001. CD8- dendritic cell activation status plays an integral role in influencing Th2 response development. *J Immunol*. 167:1982–1988.

MacDonald, A.S., A.D. Straw, N.M. Dalton, and E.J. Pearce. 2002. Cutting edge: Th2 response induction by dendritic cells: a role for CD40. *J Immunol*. 168:537–540.

MacDonald, A.S., and E.J. Pearce. 2002. Cutting edge: polarized Th cell response induction by transferred antigen-pulsed dendritic cells is dependent on IL-4 or IL-12 production by recipient cells. *J Immunol*. 168:3127–3130.

MacDonald, A.S., and R.M. Maizels. 2008. Alarming dendritic cells for Th2 induction. *J Exp Med*. 205:13–17.

MacPherson, G.G., C.D. Jenkins, M.J. Stein, and C. Edwards. 1995. Endotoxin-mediated dendritic cell release from the intestine. Characterization of released dendritic cells and TNF dependence. *J Immunol*. 154:1317–1322.

Maldonado-López, R., C. Maliszewski, J. Urbain, and M. Moser. 2001. Cytokines regulate the capacity of CD8alpha(+) and CD8alpha(-) dendritic cells to prime Th1/Th2 cells in vivo. *J Immunol*. 167:4345–4350.

Maldonado-López, R., T. De Smedt, P. Michel, J. Godfroid, B. Pajak, C. Heirman, K. Thielemans, O. Leo, J. Urbain, and M. Moser. 1999. CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J Exp Med*. 189:587–592.

Mangan, P.R., L.E. Harrington, D.B. O'Quinn, W.S. Helms, D.C. Bullard, C.O. Elson, R.D. Hatton, S.M. Wahl, T.R. Schoeb, and C.T. Weaver. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*. 441:231–234.

Mantis, N.J., N. Rol, and B. Corthésy. 2011. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol*. 4:603–611.

Masahata, K., E. Umemoto, H. Kayama, M. Kotani, S. Nakamura, T. Kurakawa, J. Kikuta, K. Gotoh, D. Motooka, S. Sato, T. Higuchi, Y. Baba, T. Kurosaki, M. Kinoshita, Y. Shimada, T. Kimura, R. Okumura, A. Takeda, M. Tajima, O. Yoshie, M. Fukuzawa, H. Kiyono, S. Fagarasan, T. Iida, M. Ishii, and K. Takeda. 2014. Generation of colonic IgA-secreting cells in the caecal patch. *Nat Commun*. 5:3704.

Massacand, J.C., R.C. Stettler, R. Meier, N.E. Humphreys, R.K. Grencis, B.J. Marsland, and N.L. Harris. 2009. Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. *Proc Natl Acad Sci U S A*. 106:13968–13973.

Mazzini, E., L. Massimiliano, G. Penna, and M. Rescigno. 2014. Oral tolerance can be established via gap junction transfer of fed antigens from CX3CR1^{+} macrophages to CD103^{+} dendritic cells. *Immunity*. 40:248–261.

McDole, J.R., L.W. Wheeler, K.G. McDonald, B. Wang, V. Konjufca, K.A. Knoop, R.D. Newberry, and M.J. Miller. 2012. Goblet cells deliver luminal antigen to CD103+ dendritic cells in the small intestine. *Nature*. 483:345–349.

Meresse, B., G. Malamut, and N. Cerf-Bensussan. 2012. Celiac disease: an immunological jigsaw. *Immunity*. 36:907–919.

Mohrs, K., A.E. Wakil, N. Killeen, R.M. Locksley, and M. Mohrs. 2005. A two-step process for cytokine production revealed by IL-4 dual-reporter mice. *Immunity*. 23:419–429.

Mountford, A.P., A. Fisher, and R.A. Wilson. 1994. The profile of IgG1 and IgG2a antibody responses in mice exposed to Schistosoma mansoni. *Parasite immunology*. 16:521–527.

Mowat, A.M., and W.W. Agace. 2014. Regional specialization within the intestinal immune system. *Nat Rev Immunol*. 14:667–685.

Naik, S.H., P. Sathe, H.-Y. Park, D. Metcalf, A.I. Proietto, A. Dakic, S. Carotta, M. O'Keeffe, M. Bahlo, A. Papenfuss, J.-Y. Kwak, L. Wu, and K. Shortman. 2007. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol*. 8:1217–1226.

Negishi, H., Y. Ohba, H. Yanai, A. Takaoka, K. Honma, K. Yui, T. Matsuyama, T. Taniguchi, and K. Honda. 2005. Negative regulation of Toll-like-receptor signaling by IRF-4. *Proc Natl Acad Sci U S A*. 102:15989–15994.

Niess, J.H., S. Brand, X. Gu, L. Landsman, S. Jung, B.A. McCormick, J.M. Vyas, M. Boes, H.L. Ploegh, J.G. Fox, D.R. Littman, and H.-C. Reinecker. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science*. 307:254–258.

Noben-Trauth, N., J. Hu-Li, and W.E. Paul. 2000. Conventional, naive CD4+ T cells provide an initial source of IL-4 during Th2 differentiation. *J Immunol*. 165:3620–3625.

Oliphant, C.J., Y.Y. Hwang, J.A. Walker, M. Salimi, S.H. Wong, J.M. Brewer, A. Englezakis, J.L. Barlow, E. Hams, S.T. Scanlon, G.S. Ogg, P.G. Fallon, and A.N.J. McKenzie. 2014. MHCII-Mediated Dialog between Group 2 Innate Lymphoid Cells and CD4+ T Cells Potentiates Type 2 Immunity and Promotes Parasitic Helminth Expulsion. *Immunity*. 41:283–295.

Pabst, O., and A.M. Mowat. 2012. Oral tolerance to food protein. *Mucosal Immunol*. 5:232–239.

Paul, W.E. 2010. What determines Th2 differentiation, in vitro and in vivo?

Immunol Cell Biol. 88:236–239.

Pearce, E.J., and A.S. MacDonald. 2002. The immunobiology of schistosomiasis. *Nat Rev Immunol*. 2:499–511.

Pearce, E.J., C. M Kane, J. Sun, J. J Taylor, A.S. McKee, and L. Cervi. 2004. Th2 response polarization during infection with the helminth parasite Schistosoma mansoni. *Immunol Rev*. 201:117–126.

Pearce, E.J., P. Caspar, J.M. Grzych, F.A. Lewis, and A. Sher. 1991. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni. *J Exp Med*. 173:159–166.

Persson, E.K., C.L. Scott, A.M. Mowat, and W.W. Agace. 2013a. Dendritic cell subsets in the intestinal lamina propria: ontogeny and function. *Eur J Immunol*. 43:3098–3107.

Persson, E.K., H. Uronen-Hansson, M. Semmrich, A. Rivollier, K. Hägerbrand, J. Marsal, S. Gudjonsson, U. Håkansson, B. Reizis, K. Kotarsky, and W.W. Agace. 2013b. IRF4 Transcription-Factor-Dependent CD103+CD11b+ Dendritic Cells Drive Mucosal T Helper 17 Cell Differentiation. *Immunity*. 38:958–969.

Phythian-Adams, A.T., P.C. Cook, R.J. Lundie, L.H. Jones, K.A. Smith, T.A. Barr, K. Hochweller, S.M. Anderton, G.U.N.J. H a mmerling, R.M. Maizels, and A.S. MacDonald. 2010. CD11c depletion severely disrupts Th2 induction and development in vivo. *J Exp Med*. 207:2089–2096.

Pieper, K., B. Grimbacher, and H. Eibel. 2013. B-cell biology and development. *J Allergy Clin Immunol*. 131:959–971.

Plantinga, M., M. Guilliams, M. Vanheerswynghels, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, H. Hammad, and B.N. Lambrecht. 2013. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity*. 38:322–335.

Pugh, C.W., G.G. MacPherson, and H.W. Steer. 1983. Characterization of nonlymphoid cells derived from rat peripheral lymph. *J Exp Med*. 157:1758–1779.

Pulendran, B., and D. Artis. 2012. New paradigms in type 2 immunity. *Science*. 337:431–435.

Pull, S.L., J.M. Doherty, J.C. Mills, J.I. Gordon, and T.S. Stappenbeck. 2005. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc Natl Acad Sci U S A*. 102:99–104.

Quah, B.J.C., H.S. Warren, and C.R. Parish. 2007. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc*. 2:2049–2056.

Ramalingam, T.R., J.T. Pesce, M.M. Mentink-Kane, S. Madala, A.W. Cheever, M.R. Comeau, S.F. Ziegler, and T.A. Wynn. 2009. Regulation of helminth-induced Th2 responses by thymic stromal lymphopoietin. *J Immunol*. 182:6452–6459.

Randolph, G.J., V. Angeli, and M.A. Swartz. 2005. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat Rev Immunol*. 5:617–628.

Read, S., V. Malmström, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*. 192:295–302.

Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J.P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol*. 2:361–367.

Robertson, J.M., P.E. Jensen, and B.D. Evavold. 2000. DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J Immunol*. 164:4706–4712.

Romani, N., S. Koide, M. Crowley, M. Witmer-Pack, A.M. Livingstone, C.G. Fathman, K. Inaba, and R.M. Steinman. 1989. Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells. *J Exp Med*. 169:1169–1178.

Rubtsov, Y.P., J.P. Rasmussen, E.Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W.R. Henderson, W. Müller, and A.Y. Rudensky. 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*. 28:546–558.

Saito, M., T. Iwawaki, C. Taya, H. Yonekawa, M. Noda, Y. Inui, E. Mekada, Y. Kimata, A. Tsuru, and K. Kohno. 2001. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotechnol*. 19:746–750.

Satoh, T., O. Takeuchi, A. Vandenbon, K. Yasuda, Y. Tanaka, Y. Kumagai, T. Miyake, K. Matsushita, T. Okazaki, T. Saitoh, K. Honma, T. Matsuyama, K. Yui, T. Tsujimura, D.M. Standley, K. Nakanishi, K. Nakai, and S. Akira. 2010. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol*. 11:936–944.

Satpathy, A.T., C.G. Briseño, J.S. Lee, D. Ng, N.A. Manieri, W. KC, X. Wu, S.R. Thomas, W.-L. Lee, M. Turkoz, K.G. McDonald, M.M. Meredith, C. Song, C.J. Guidos, R.D. Newberry, W. Ouyang, T.L. Murphy, T.S. Stappenbeck, J.L. Gommerman, M.C. Nussenzweig, M. Colonna, R. Kopan, and K.M. Murphy. 2013. Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching-and-effacing bacterial pathogens. *Nat Immunol*. 14:937–948.

Schlitzer, A., N. McGovern, P. Teo, T. Zelante, K. Atarashi, D. Low, A.W.S. Ho, P. See, A. Shin, P.S. Wasan, G. Hoeffel, B. Malleret, A. Heiseke, S. Chew, L. Jardine, H.A. Purvis, C.M.U. Hilkens, J. Tam, M. Poidinger, E.R. Stanley, A.B. Krug, L. Renia, B. Sivasankar, L.G. Ng, M. Collin, P. Ricciardi-Castagnoli, K. Honda, M. Haniffa, and F. Ginhoux. 2013. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity*. 38:970–983.

Schramm, G., K. Mohrs, M. Wodrich, M.J. Doenhoff, E.J. Pearce, H. Haas, and M. Mohrs. 2007. cutting edge: IPSE/alpha-1, a glycoprotein from Schistosoma mansoni eggs, induces IgE-dependent, antigen-independent IL-4 production by

murine basophils in vivo. *J Immunol*. 178:6023–6027.

Schuler, G., and R.M. Steinman. 1985. Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J Exp Med*. 161:526–546.

Schulz, O., E. Jaensson, E.K. Persson, X. Liu, T. Worbs, W.W. Agace, and O. Pabst. 2009. Intestinal CD103+, but not CX3CR1+, antigen sampling cells migrate in lymph and serve classical dendritic cell functions. *J Exp Med*. 206:3101–3114.

Scott, C.L., P.B. Wright, S.W.F. Milling, and A.M. Mowat. 2016. Isolation and Identification of Conventional Dendritic Cell Subsets from the Intestine of Mice and Men. *Methods Mol Biol*. 1423:101–118.

Seshasayee, D., W.P. Lee, M. Zhou, J. Shu, E. Suto, J. Zhang, L. Diehl, C.D. Austin, Y.G. Meng, M. Tan, S.L. Bullens, S. Seeber, M.E. Fuentes, A.F. Labrijn, Y.M.F. Graus, L.A. Miller, E.S. Schelegle, D.M. Hyde, L.C. Wu, S.G. Hymowitz, and F. Martin. 2007. In vivo blockade of OX40 ligand inhibits thymic stromal lymphopoietin driven atopic inflammation. *J Clin Invest*. 117:3868–3878.

Silva-Moraes, V., J.M.S. Ferreira, P.M.Z. Coelho, and R.F.Q. Grenfell. 2014. Biomarkers for schistosomiasis: Towards an integrative view of the search for an effective diagnosis. *Acta Tropica*. 132:75–79.

Smith, K.A., K. Hochweller, G.J. Hämmerling, L. Boon, A.S. MacDonald, and R.M. Maizels. 2011. Chronic helminth infection promotes immune regulation in vivo through dominance of CD11cloCD103- dendritic cells. *J Immunol*. 186:7098–7109.

Smits, H.H., H. Hammad, M. van Nimwegen, T. Soullie, M.A. Willart, E. Lievers, J. Kadouch, M. Kool, J. Kos-van Oosterhoud, A.M. Deelder, B.N. Lambrecht, and M. Yazdanbakhsh. 2007. Protective effect of Schistosoma mansoni infection on allergic airway inflammation depends on the intensity and chronicity of infection. *J Allergy Clin Immunol*. 120:932–940.

Smythies, L.E., C. Betts, P.S. Coulson, M.A. Dowling, and R.A. Wilson. 1996. Kinetics and mechanism of effector focus formation in the lungs of mice vaccinated with irradiated cercariae of Schistosoma mansoni. *Parasite Immunol*. 18:359–369.

Smythies, L.E., M. Sellers, R.H. Clements, M. Mosteller-Barnum, G. Meng, W.H. Benjamin, J.M. Orenstein, and P.D. Smith. 2005. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest*. 115:66–75.

Soumelis, V., P.A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S. Ho, S. Antonenko, A. Lauerma, K. Smith, D. Gorman, S. Zurawski, J. Abrams, S. Menon, T. McClanahan, R. de Waal-Malefyt Rd, F. Bazan, R.A. Kastelein, and Y.-J. Liu. 2002. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol*. 3:673–680.

Spits, H., and T. Cupedo. 2012. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol*. 30:647–675.

Steinfelder, S., J.F. Andersen, J.L. Cannons, C.G. Feng, M. Joshi, D. Dwyer, P. Caspar, P.L. Schwartzberg, A. Sher, and D. Jankovic. 2009. The major component

in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). *J Exp Med*. 206:1681–1690.

Steinman, R.M., and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med*. 137:1142–1162.

Stone, K.D., C. Prussin, and D.D. Metcalfe. 2010. IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol*. 125:S73–80.

Suzuki, K., B. Meek, Y. Doi, M. Muramatsu, T. Chiba, T. Honjo, and S. Fagarasan. 2004a. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. *Proc Natl Acad Sci U S A*. 101:1981–1986.

Suzuki, S., K. Honma, T. Matsuyama, K. Suzuki, K. Toriyama, I. Akitoyo, K. Yamamoto, T. Suematsu, M. Nakamura, K. Yui, and A. Kumatori. 2004b. Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development. *Proc Natl Acad Sci U S A*. 101:8981–8986.

Szabo, S.J., S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, and L.H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*. 100:655–669.

Tal, O., H.Y. Lim, I. Gurevich, I. Milo, Z. Shipony, L.G. Ng, V. Angeli, and G. Shakhar. 2011. DC mobilization from the skin requires docking to immobilized CCL21 on lymphatic endothelium and intralymphatic crawling. *J Exp Med*. 208:2141–2153.

Talham, G.L., H.Q. Jiang, N.A. Bos, and J.J. Cebra. 1999. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect Immun*. 67:1992–2000.

Taylor, B.C., C. Zaph, A.E. Troy, Y. Du, K.J. Guild, M.R. Comeau, and D. Artis. 2009. TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med*. 206:655–667.

Tjota, M.Y., C.L. Hrusch, K.M. Blaine, J.W. Williams, N.A. Barrett, and A.I. Sperling. 2014. Signaling through FcRγ-associated receptors on dendritic cells drives IL-33-dependent TH2-type responses. *J Allergy Clin Immunol*. 134:706–713.e8.

Uematsu, S., K. Fujimoto, M.H. Jang, B.-G. Yang, Y.-J. Jung, M. Nishiyama, S. Sato, T. Tsujimura, M. Yamamoto, Y. Yokota, H. Kiyono, M. Miyasaka, K.J. Ishii, and S. Akira. 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat Immunol*. 9:769–776.

Urb, M., and D.C. Sheppard. 2012. The role of mast cells in the defence against pathogens. *PLoS Pathog*. 8:e1002619.

Urban, J.F., N. Noben-Trauth, D.D. Donaldson, K.B. Madden, S.C. Morris, M. Collins, and F.D. Finkelman. 1998. IL-13, IL-4Ralpha, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite Nippostrongylus brasiliensis. *Immunity*. 8:255–264.

van der Werf, M.J., S.J. de Vlas, S. Brooker, C.W.N. Looman, N.J.D. Nagelkerke,

J.D.F. Habbema, and D. Engels. 2003. Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. *Acta Tropica*. 86:125–139.

van Liempt, E., S.J. van Vliet, A. Engering, J.J. García Vallejo, C.M.C. Bank, M. Sanchez-Hernandez, Y. van Kooyk, and I. van Die. 2007. Schistosoma mansoni soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. *Mol Immunol*. 44:2605–2615.

van Rijt, L.S., S. Jung, A. Kleinjan, N. Vos, M. Willart, C. Duez, H.C. Hoogsteden, and B.N. Lambrecht. 2005. In vivo depletion of lung CD11c+ dendritic cells during allergen challenge abrogates the characteristic features of asthma. *J Exp Med*. 201:981–991.

Vander Lugt, B., A.A. Khan, J.A. Hackney, S. Agrawal, J. Lesch, M. Zhou, W.P. Lee, S. Park, M. Xu, J. DeVoss, C.J. Spooner, C. Chalouni, L. Delamarre, I. Mellman, and H. Singh. 2014. Transcriptional programming of dendritic cells for enhanced MHC class II antigen presentation. *Nat Immunol*. 15:161–167.

Varol, C., A. Vallon-Eberhard, E. Elinav, T. Aychek, Y. Shapira, H. Luche, H.J. Fehling, W.-D. Hardt, G. Shakhar, and S. Jung. 2009. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity*. 31:502–512.

Veenbergen, S., L.A. van Berkel, M.F. du Pré, J. He, J.J. Karrich, L.M.M. Costes, F. Luk, Y. Simons-Oosterhuis, H.C. Raatgeep, V. Cerovic, T. Cupedo, A.M. Mowat, B.L. Kelsall, and J.N. Samsom. 2016. Colonic tolerance develops in the iliac lymph nodes and can be established independent of CD103(+) dendritic cells. *Mucosal Immunol*. 9:894–906.

Vennervald, B.J., and K. Polman. 2009. Helminths and malignancy. *Parasite Immunol*. 31:686–696.

Villadangos, J.A., P. Schnorrer, and N.S. Wilson. 2005. Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunol Rev*. 207:191–205.

Vremec, D., and K. Shortman. 1997. Dendritic cell subtypes in mouse lymphoid organs: cross-correlation of surface markers, changes with incubation, and differences among thymus, spleen, and lymph nodes. *J Immunol*. 159:565–573.

Waskow, C., K. Liu, G. Darrasse-Jèze, P. Guermonprez, F. Ginhoux, M. Merad, T. Shengelia, K. Yao, and M. Nussenzweig. 2008. The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat Immunol*. 9:676–683.

Weaver, C.T., L.E. Harrington, P.R. Mangan, M. Gavrieli, and K.M. Murphy. 2006. Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity*. 24:677–688.

Williams, J.W., M.Y. Tjota, B.S. Clay, B. Vander Lugt, H.S. Bandukwala, C.L. Hrusch, D.C. Decker, K.M. Blaine, B.R. Fixsen, H. Singh, R. Sciammas, and A.I. Sperling. 2013. Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nat Commun*. 4:2990.

Wills-Karp, M. 2004. Interleukin-13 in asthma pathogenesis. *Immunol Rev*. 202:175–190.

Wilson, M.S., M.M. Mentink-Kane, J.T. Pesce, T.R. Ramalingam, R. Thompson, and T.A. Wynn. 2007. Immunopathology of schistosomiasis. *Immunol Cell Biol*. 85:148–154.

Worbs, T., U. Bode, S. Yan, M.W. Hoffmann, G. Hintzen, G. Bernhardt, R. Förster, and O. Pabst. 2006. Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med*. 203:519–527.

Wynn, T.A., R.W. Thompson, A.W. Cheever, and M.M. Mentink-Kane. 2004. Immunopathogenesis of schistosomiasis. *Immunol Rev*. 201:156–167.

Yamane, H., J. Zhu, and W.E. Paul. 2005. Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. *J Exp Med*. 202:793–804.

Yashiro, T., M. Hara, H. Ogawa, K. Okumura, and C. Nishiyama. 2016. Critical Role of Transcription Factor PU.1 in the Function of the OX40L/TNFSF4 Promoter in Dendritic Cells. *Sci Rep*. 6:34825.

Yokota-Nakatsuma, A., H. Takeuchi, Y. Ohoka, C. Kato, S.-Y. Song, T. Hoshino, H. Yagita, T. Ohteki, and M. Iwata. 2014. Retinoic acid prevents mesenteric lymph node dendritic cells from inducing IL-13-producing inflammatory Th2 cells. *Mucosal Immunol*. 7:786–801.

Yoshimoto, T. 2010. Basophils as T(h)2-inducing antigen-presenting cells. *Int Immunol*. 22:543–550.

You, H., D.P. McManus, W. Hu, M.J. Smout, P.J. Brindley, and G.N. Gobert. 2013. Transcriptional responses of in vivo praziquantel exposure in schistosomes identifies a functional role for calcium signalling pathway member CamKII. *PLoS Pathog*. 9:e1003254.

Yrlid, U., S.W.F. Milling, J.L. Miller, S. Cartland, C.D. Jenkins, and G.G. MacPherson. 2006. Regulation of intestinal dendritic cell migration and activation by plasmacytoid dendritic cells, TNF-alpha and type 1 IFNs after feeding a TLR7/8 ligand. *J Immunol*. 176:5205–5212.

Zaccone, P., O. Burton, N. Miller, F.M. Jones, D.W. Dunne, and A. Cooke. 2009. Schistosoma mansoni egg antigens induce Treg that participate in diabetes prevention in NOD mice. *Eur J Immunol*. 39:1098–1107.

Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J.M. Kim, T.-T. Chu, L. Corcoran, P. Treuting, U. Klein, and A.Y. Rudensky. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature*. 458:351–356.

Zhou, B., M.R. Comeau, T. De Smedt, H.D. Liggitt, M.E. Dahl, D.B. Lewis, D. Gyarmati, T. Aye, D.J. Campbell, and S.F. Ziegler. 2005. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol*. 6:1047–1053.

Zhu, J., and W.E. Paul. 2008. CD4 T cells: fates, functions, and faults. *Blood*.

112:1557–1569.

Ziegler, S.F., and D. Artis. 2010. Sensing the outside world: TSLP regulates barrier immunity. *Nat Immunol*. 11:289–293.

Zoetendal, E.G., E.E. Vaughan, and W.M. de Vos. 2006. A microbial world within us. *Mol Microbiol*. 59:1639–1650.