REGULATION OF INTESTINAL INFLAMMATION BY CD45

by

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Abstract

Factors secreted by intestinal immune cells such as retinoic acid and cytokines are crucial in maintaining homeostasis in the gut. Dysregulation in the secretion of these factors can lead to inflammation and development of colitis. CD45 is a leukocyte specific tyrosine phosphatase important for T cell development and antigen receptor signaling. Here, I show that upon DSS-induced colitis, CD45$^{-/-}$ mice unexpectedly have significant numbers of T cells in their colon and exhibit more severe colitis. This was attributed to increased expression of the gut homing molecule, $\alpha 4\beta 7$ and increased production of IFN$\gamma$ and IL17A by the CD45$^{-/-}$ T cells. However, in the absence of adaptive immunity, CD45 is required for optimal intestinal innate immune responses. CD45$^{-/-}$ innate lymphoid cells had decreased IL-22 and GM-CSF production and CD45$^{-/-}$ myeloid cells have lower retinoic acid production. This led to less severe colitis when CD45$^{+/+}$ T cells were transferred into CD45RAG$^{-/-}$ mice as it led to reduced expression of gut homing molecules and reduced homing of T cells to the colon. This defect was corrected by the addition of GM-CSF, which restored retinoic acid production.

Induction of colitis by the transfer of naïve T cells into RAG$^{-/-}$ and CD45RAG$^{-/-}$ mice delayed the development of systemic disease in CD45RAG$^{-/-}$ mice, but led to comparable intestinal inflammation at the RAG$^{-/-}$ weight loss endpoint and significantly greater inflammation at the CD45RAG$^{-/-}$ endpoint, corresponding with increased CD45$^{-/-}$ myeloid cells in the colon. Since there was no difference in Foxp3+ regulatory T cells systemically, other options of inhibition of systemic inflammation in CD45RAG$^{-/-}$ mice were explored. CD45RAG$^{-/-}$ mice had increased CD71+TER119+ erythroid cells in the spleen prior to and post colitis induction and failed to downregulate erythroid progenitors upon T cell induced colitis. These suppressive erythroid cells may contribute to the delayed systemic inflammation in these mice. Overall, these
results show novel roles for CD45 in the regulation of innate and adaptive immune cell cytokine production, as well as in erythrocyte maturation.
Preface

I conducted all of the experimental research and analyses, with the following exceptions:

- Manisha Dosanjh helped with the cell preparation of spleen and lymph nodes
- Camille Labonte-Raymond, an undergraduate under my mentorship was responsible for the experiments and analysis for Figure 4.12
- Michael Crickmer, an undergraduate under my mentorship was responsible for some of the experiments and analysis in Figure 3.8A

A version of Chapter 3 forms a manuscript that has been submitted for publication and is now in revision.

A version of Chapter 4 is now part of a manuscript in preparation to be submitted for publication.

Animal experimentation was conducted in accordance with protocols approved by the University of British Columbia Animal Care Committee and Canadian Council of Animal Care guidelines. Project titles and certificate numbers applicable to this project include:

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CD44 and CD45 breeding protocol number: A08-0936
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Regulation of Signaling and Dendritic Cell Function by CD45, A07-0786
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List of Symbols

α  alpha
β  beta
δ  delta
γ  gamma
μ  micro
ζ  zeta
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ACD</td>
<td>anemia of chronic diseases</td>
</tr>
<tr>
<td>BMDC</td>
<td>bone marrow derived dendritic cell</td>
</tr>
<tr>
<td>BMDM</td>
<td>bone marrow derived macrophages</td>
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<tr>
<td>CCL</td>
<td>C-C chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CD45KO</td>
<td>CD45 deficient mice</td>
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<tr>
<td>cDC</td>
<td>conventional dendritic cell</td>
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<tr>
<td>CX3CR1</td>
<td>C-X3- chemokine receptor 1</td>
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<td>danger associated molecular pattern</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>GALT</td>
<td>gut-associated lymphoid tissues</td>
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<td>granulocyte and macrophage colony stimulating factor</td>
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<td>IBD</td>
<td>inflammatory bowel disease</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>ICS</td>
<td>intracellular stain</td>
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<td>JAK</td>
<td>janus kinase</td>
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<tr>
<td>KO</td>
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<td>LI</td>
<td>large intestine</td>
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<td>LPS</td>
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<td>MAdCAM-1</td>
<td>mucosal vascular addressin cell adhesion molecule 1</td>
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<td>macrophage colony stimulating factor</td>
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<tr>
<td>MHCII</td>
<td>major histocompatibility complex II</td>
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<td>myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PRR</td>
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<td>RAG</td>
<td>recombination activation gene</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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</tr>
<tr>
<td>RORα</td>
<td>RAR-related orphan receptor alpha</td>
</tr>
<tr>
<td>RORγt</td>
<td>retinoic acid related orphan receptor gamma t</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SI</td>
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</tr>
<tr>
<td>SFK</td>
<td>src family kinase</td>
</tr>
<tr>
<td>SLO</td>
<td>secondary lymphoid organ</td>
</tr>
<tr>
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<td>signal transducer and activator of transcription</td>
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<tr>
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<td>T cell receptor</td>
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<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
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<td>Th1</td>
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<td>T helper 2</td>
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<tr>
<td>Th17</td>
<td>T helper 17</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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<td>WT</td>
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Chapter 1: Introduction

1.1 Overview of the Immune System

The immune system is a network of cells, secreted factors and cytokines and has multiple ways in protecting the host. The immune system works to defend the host from any pathogen that gains entry, heal upon injury and also maintain tolerance to the trillions of beneficial commensal flora in the host. The immune system consists of both an innate and adaptive component.

The innate immune system is considered the first line of defense against a pathogen where cells such as myeloid antigen presenting cells (APCs) including dendritic cells (DCs) and macrophages express receptors that can detect characteristics of a pathogen including pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). DCs are a critical link between the innate and adaptive immune system because when they recognize a pathogen, they migrate to secondary lymphoid organs (SLO) such as lymph nodes and activate naïve T cells into effector T cells, thus providing a bridge between the innate and adaptive immune system.

When the innate immune system is not sufficient to contain the pathogen, the adaptive immune system consisting of T and B lymphocytes become activated. T and B cells recognize antigens that have been directed to SLOs. An antigen is recognized by these lymphocytes through their antigen receptors, the T cell receptor (TCR) and the B cell receptor, respectively. Once antigen recognition occurs, T cells and B cells are activated, differentiate and proliferate to become effector cells. The importance of the adaptive immune system in pathogen clearance is demonstrated in mice that lack T and B cells such as severe combined immunodeficiency (SCID).
or recombination activation gene (RAG) deficient mice, which succumb to infection with viruses and bacteria (Bosma and Carroll, 1991; Mombaerts et al., 1992).

While the innate and adaptive immune system have distinctive roles in protecting the host, a key feature of both adaptive and innate immune cells is that they communicate with themselves, each other and non-hematopoietic cells through cell-cell contact and the secretion of soluble factors in order to effectively defend.

1.2 Overview of the Intestinal Immune System

The intestinal tract is an internal extension of the body’s surface and as such is in constant exposure to environmental stimuli that includes pathogens, food particles and commensal bacteria (Sansonetti, 2004). Remarkably, the separation of the environment from the host in the intestine is only by a single layer of epithelial cells and in the large and small bowel, the mucosal tissue is in continuous contact with over 100 trillion microbiota (Yan and Polk, 2004). While a major role of the immune system is to contain the microbes and prevent their systemic spread, commensals themselves have been shown to have distinct influences on the immune system such as promoting its development and inducing specific regulatory function. For instance, the human symbiotic microbe, Bacteroides fragilis, produces polysaccharide A (PSA) and can protect mice from colitis, which was shown to coincide with the capacity of PSA to induce and expand regulatory T cells that produced IL-10 (Mazmanian et al., 2008). In addition to inducing immune regulatory function, the intestinal microbiota also has other beneficial effects on the host such as colonization resistance to exogenous pathogens through competition for metabolites or production of antimicrobial peptides and the capacity to facilitate digestion (Buffie and Pamer, 2013; Hammami et al., 2013; Kamada et al., 2013).
While the microbiota is known to exert its influence on the immune system, cells of both the adaptive and innate immune system must also be able to contain the microbiota, maintain a state of tolerance and prevent unnecessary inflammation (Belkaid and Hand, 2014). Cells of the intestinal immune system include myeloid cells such as macrophages and dendritic cells that respond to environmental cues. Dendritic cells and macrophages in turn can direct the responses of other innate immune cells such as innate lymphoid cells to secrete particular cytokines. Subsets of dendritic cells also direct the adaptive immune system by inducing the conversion of naïve T cells into regulatory T cells or the various effector T cells subsets as well as directing B cells to secrete IgA.

Importantly, these cells of the intestinal immune system must also be able to mount an appropriate response to a pathogen and if the balance between the active state of tolerance in the intestine and the capacity to mount an effective inflammatory response breaks down, diseases such as colitis may result.

### 1.2.1 Organization and Structure

The commensal bacteria in the intestine are not in direct contact with the epithelium below them. A mucous layer known as the glycocalyx, which is thickest in the colon, separates them. The glycocalyx consists of two layers and while bacteria can penetrate the outer layer, they are less likely to be found in the denser inner layer (Johansson et al., 2008). The lamina propria (LP) along with the epithelium are the effector sites of the intestinal immune system. The LP is a loose connective tissue underneath the epithelium where the majority of the immune cells reside. Induction of effector responses occur in the gut associated lymphoid tissues (GALT). Components of the GALT include, Peyer’s patches, microfold (M) cells, isolated lymphoid
follicles and lymph nodes that drain the gut. Peyer’s patches localized in the small intestine (SI) and isolated lymphoid follicles in the large intestine (LI) are sites of germinal centers where B cells mature and proliferate into IgA producing plasma cells (Mowat and Agace, 2014). M cells are specialized epithelial cells in the Peyer’s patches that transport antigens from the lumen often to DCs directly below them (Gebert et al., 1996). The majority of the large and small intestine are drained by the mesenteric and caudal lymph nodes (Carter and Collins, 1974).

1.2.2 Macrophages

Macrophages are traditionally defined as tissue resident mononuclear phagocytes that are inefficient at activating naïve T cells. Phenotypically, intestinal macrophages express the surface molecules CD11c, CX3CR1 and MHCII that are also expressed by DCs in the gut (Bain and Mowat, 2014). While DCs and macrophages share similar phenotypic markers, functionally, there are differences. Intestinal macrophages in steady-state are in close association with the epithelium and have trans epithelial dendrites that have been shown to sample the luminal bacteria (Rescigno et al., 2001; Vallon-Eberhard et al., 2006). Although steady-state intestinal macrophages express pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), recognition of the intestinal microbiota does not trigger an inflammatory response and while they are phagocytic, they also do not express reactive oxygen species or nitric oxide (Roberts et al., 2001). Instead intestinal macrophages are thought to secrete the regulatory cytokine, IL-10 and interestingly they also express the IL-10 receptor and respond to this regulatory cytokine in an autocrine manner in order to conduct their anti-inflammatory function (Hoshi et al., 2012; Shouval et al., 2014; Zigmond et al., 2014). The production of IL-10 by macrophages in addition to acting in an autocrine manner may also maintain the pool of regulatory T cells (Tregs) in the
gut compounding the tolerogenic state and/or preventing excessive inflammatory responses (Kayama et al., 2012). Intestinal macrophages have also been shown to produce other regulatory factors such as arginase and inhibit the inflammatory neutrophils via prostaglandin E2 during *Toxoplasma gondii* infection (Grainger et al., 2013). While steady-state macrophages do secrete large amounts of IL-10, they also secrete TNFα and IL-1β during homeostasis, likely to maintain the barrier and production of retinoic acid (RA) by intestinal DCs (Bain et al., 2013; Mortha et al., 2014).

While other tissue macrophages such as microglia in the central nervous system, alveolar macrophages in the lung and Kupffer cells in the liver are derived from progenitors from the yolk sac or fetal liver determined by lineage tracking, intestinal macrophages are not (Bain and Mowat, 2014; Hashimoto et al., 2013; Yona et al., 2013). Instead, gut macrophages have been shown to be derived from Ly6C^hi^ monocytes even in steady-state (Varol et al., 2009). Ly6C^hi^ monocytes themselves are derived from the common macrophage dendritic cell progenitor (MDP) in the bone marrow and these monocytes exit the bone marrow in a CCR2 dependent manner (Fogg et al., 2006; Varol et al., 2007). However, not all intestinal macrophages are derived from the LyC^hi^ monocytes since CCR2 deficient mice lack Ly6C^hi^ monocytes but still retain some intestinal macrophages suggesting that there is an additional source of these cells (Takada et al., 2010).

While there are attempts to distinguish intestinal DCs and macrophages from one another by characterizing DCs as CX3CR1 (fractalkine receptor) intermediate (CX3CR1^int^) with an ability to migrate to lymph nodes and macrophages as CX3CR1 high (C3CR1^hi^) with an inability to migrate from the gut or activate naïve T cells, there are conflicting reports to these definitions (Schulz et al., 2009; Zigmond and Jung, 2013). Diehl et al. have reported that
CX3CR1^{hi} cells can migrate to the mesenteric lymph nodes (mLN) in a CCR7 dependent manner in the absence of MyD88 while Schulz et al. show that only CD103^{+} DCs migrate and not CX3CR1 positive cells in a model of oral tolerance (Diehl et al., 2013; Schulz et al., 2009). Interestingly it has recently been shown that CX3CR1^{hi} macrophages can take up soluble antigen and transfer them to the CD103^{+} DCs via the gap junction, connexin 43 (Mazzini et al., 2014). The process also involves membrane transfer between the two cell types so it is possible that through trogocytosis, CD103^{+} DCs acquire CX3CR1 during this process making them phenotypically similar to macrophages (Mazzini et al., 2014).

Upon inflammation, it is even less clear whether the myeloid cells in the colon are Ly6C^{hi} derived macrophages or DCs or resident macrophages that have switched to become inflammatory. Using extensive phenotypic and gene array analysis, Rivollier et al. concluded that in a mouse model of colitis, inflammation switches Ly6C^{hi} monocytes from becoming anti-inflammatory macrophages in the intestine to inflammatory DCs (Rivollier et al., 2012). Other groups have also shown that the majority of the myeloid cells in the colon switch from being CX3CR1^{high} macrophages in homeostasis to Cx3CR1^{int} DCs upon inflammation (Bain et al., 2013; Zigmond et al., 2012). There is also evidence that resident CX3CR1^{hi} macrophages retain their ability to secrete IL-10 in colitis and may prevent excessive pro-inflammatory action by DCs (Bain et al., 2013). Alternatively, another report suggests that monocytes become inflammatory intestinal macrophages upon T cell colitis and make inducible nitric oxide synthase (Tamoutounour et al., 2012). Taken together, these results suggest that the majority of cells recruited to the colon upon inflammation are inflammatory DCs derived from Ly6C^{hi} monocytes, however, it is still not conclusive that intestinal macrophages don’t also play an inflammatory role upon inflammation.
1.2.3 Dendritic Cells

In steady-state, DCs have distinct roles in the intestine. DCs are a smaller proportion of the myeloid cells in the gut but are critical cells in maintaining homeostasis. Intestinal DCs are a heterogeneous population and of the CD11c<sup>hi</sup>MHCII<sup>hi</sup> cells in the gut, they can be characterized as CD103<sup>+</sup>CD11b<sup>+</sup>, CD103<sup>+</sup>CD11b<sup>-</sup>, CD103<sup>-</sup>CD11b<sup>+</sup> and CD103<sup>-</sup>CD11b<sup>-</sup> (Bekiaris et al., 2014). CD103 is the αE integrin that associates with the β7 subunit; its most common ligand is E-cadherin, which is expressed on the basolateral side of intestinal epithelial cells (Agace et al., 2000). CD103 is also expressed on T cells and one role for this integrin is to retain cells in the intestine (Schon et al., 1999; Strauch et al., 2001).

CD103<sup>-</sup>CD11b<sup>+</sup> DCs express intermediate levels of CX3CR1 and macrophages are also included in this CD103<sup>-</sup>CD11b<sup>+</sup> subset. There is little known about the CD103<sup>-</sup>CD11b<sup>-</sup> subset but they are present in both the small and large intestine. The CD103<sup>+</sup> DCs are the most characterized subset in the gut and they are found to be key contributors to the maintenance of homeostasis in the gut. The small and large intestine have different proportions of CD103<sup>+</sup> DCs; the small intestine has a larger proportion of CD103<sup>-</sup>CD11b<sup>+</sup> DCs, while the large intestine has more CD103<sup>-</sup>CD11b<sup>-</sup> DCs (Bekiaris et al., 2014).

1.2.3.1 Intestinal CD103+ DCs

In steady-state, the CD103<sup>+</sup> DCs are thought to be derived primarily from pre-DC progenitors, however there are discrepancies in identifying the sole progenitor for these cells. Pre-DCs are progenitors that are found in the blood and tissues and can differentiate into mature DC subsets in the tissues (Naik et al., 2007; Onai et al., 2007). Pre-DCs themselves are derived
from the common dendritic cell progenitor (CDP) that can become either pre-DCs or plasmacytoid DCs (Naik et al., 2007; Onai et al., 2007). Adoptive transfer studies have suggested that both pre-DCs and Ly6C<sup>hi</sup> monocytes both contribute to the development of the CD103<sup>+</sup> subset. To define and track subsets of DCs that are of the classical DC lineage and not monocyte-derived, plasmacytoid DCs or other immune cells, Meredith et al. identified the zinc finger transcription factor, Zbtb46 expression in only pre-DC derived DCs (Meredith et al., 2012). A subsequent study using depletion of Zbtb46 expressing cells in mice, found that upon depletion, the intestinal CD11b<sup>+</sup>CD103<sup>+</sup> subset was only partially reduced while the CD103<sup>-</sup>CD11b<sup>-</sup> subset was completely depleted. The same study using mice where monocytes could be depleted, again CD103<sup>-</sup>CD11b<sup>+</sup> cells were partially depleted (Schreiber et al., 2013).

Currently, there is still no guaranteed method to identify the sole progenitors for DCs and macrophages in the intestine or even the inflammatory intestinal myeloid cell type. Therefore, further studies will be crucial in identifying these cells and distinguishing between macrophages and DCs.

1.2.3.2 Antigen Uptake by Intestinal DCs

One way for DCs to acquire antigen is via M cells present in the subepithelial dome of Peyer’s patches. M cells will transcytose luminal antigens to DCs in this region (Neutra et al., 1999). Goblet cells also function as routes for the delivery of low molecular weight soluble antigens from the lumen to DCs in the LP (McDole et al., 2012). When antigen is acquired, CD103+ DCs migrate to the draining lymph nodes in a CCR7 dependent manner (Jang et al., 2006; Schulz et al., 2009).
1.2.4 The Diverse Roles of RA in the Intestinal Immune System

Once CD103+ DCs arrive at the mLNs, they secrete RA and TGFβ, which create a milieu for the conversion of naïve T cells into Tregs (Coombes et al., 2007; Denning et al., 2007; Worthington et al., 2011). The secretion of RA also imprints these Tregs with the gut homing molecules, α4β7 and CCR9 which enables Tregs to home to the gut to exert their regulatory function (Benson et al., 2007; Iwata et al., 2004; Kang et al., 2007). RA is the active metabolite derived from dietary vitamin A also known as retinol. CD103+ DCs are uniquely equipped with the enzymes, retinal dehydrogenases, to covert retinol into RA (Coombes et al., 2007). RA can act on DCs themselves in an autocrine manner to promote further RA production (Zhu et al., 2013). In addition, the cytokines GM-CSF and IL-4 as well as TLR signaling can also enhance RA signaling (Mortha et al., 2014; Yokota et al., 2009). The tolerogenic effects of RA are also evident upon inflammation. In SAMP1/Yit mice that develop spontaneous ileitis, provision of enhanced vitamin A induced Tregs in these mice and these same Tregs ameliorated inflammation upon transfer and induction of T cell induced colitis (Kang et al., 2009). Furthermore, upon dextran sodium sulphate (DSS) induced colitis, RA ameliorates disease by acting on ILCs and γδ T cells to induce their production of the cytokine IL-22, which has been shown to be important for the maintenance of the epithelial barrier (Mielke et al., 2013). Interestingly, upon infection, RA contributes to the clearance of the pathogen by inducing homing molecules on effector T cells (Hall et al., 2011). Paradoxically, providing a vitamin A deficient diet to the SAMP1/Yit mice that develop spontaneous ileitis also enhances Tregs and T cell induced colitis is ameliorated. Furthermore, the transfer of T cells that are deficient for the RA-degrading enzyme, CYP26B1 are protected against T cell induced intestinal inflammation, suggesting that RA and its signaling can attenuate inflammation as well (Chenery et al., 2013; Kang et al., 2009). In
addition, inhibiting RA signaling in CD8+ effector T cells reduces their numbers and decreases expression of gut homing molecules on these cells (Svensson et al., 2008). Therefore, the roles of RA upon steady-state are to maintain tolerance and contribute to immune homeostasis and upon inflammation are to maintain the barrier and promote clearance of infection as summarized in (Figure 1.1). Furthermore, RA is an example of a soluble factor produced by various cell-types including DCs and stromal cells that can communicate with a range of other immune cells in order to effectively trigger an immune response (Vicente-Suarez et al., 2015).
Figure 1.1 The immunomodulatory roles of RA in steady state and inflammation (Raverdeau and Mills, 2014). In steady state, RA produced by myeloid cells induces conversion of naïve T cells into Tregs and inhibits pro-inflammation effector T cell generation. Upon inflammation, RA induces homing molecules on effector T cells and IL-22 production by γδ T cells and ILCs.
1.2.5 Innate Lymphoid Cells

ILCs are a group of cells that have recently been identified and are primarily found at mucosal barrier surfaces. ILCs can be classified into Group 1, 2 and 3 ILCs based mainly on the different cytokines they secrete and the transcription factors required for their development (Spits et al., 2013). Group 1 ILCs (ILC1) requires the transcription factor, T-bet and secrete IFNγ in response to IL-12, IL-15 and likely IL-23 (Daussy et al., 2014; Fuchs et al., 2013; Klose et al., 2013). Group 2 ILCs secrete type 2 cytokines such as IL-5 and IL-13 in response to IL-25, IL-33 and/or thymic stromal lymphopoietin (TSLP) (Koyasu and Moro, 2012). ILC2s are crucial for the clearance of certain helminth infections and secrete similar type 2 cytokines as Th2 effector T cells (Fort et al., 2001). Group 2 ILCs require the transcription factors, retinoic acid related orphan receptor α (RORα) and GATA3 for development (Halim et al., 2012; Hoyler et al., 2012). ILC3s are classified in the third group of ILCs, require the transcription factor, retinoic acid related orphan receptor γ (RORγt) and secrete the cytokines IL-22 and IL-17A (Cella et al., 2009; Cupedo et al., 2009).

While ILCs are considered part of the innate immune system, upon inflammation or infection, ILCs continually communicate with other cells to promote clearance of the infection or perpetuate inflammation. In one study, Oliphant et al. show that ILC2s express MHCII and interact with antigen-specific T cells to promote IL-2 production from T cells. In an infection with the worm, Nippostrongylus brasiliensis, this interaction was shown to be crucial for the clearance on this intestinal pathogen (Oliphant et al., 2014). Alternatively, Mortha et al. describes the communication between ILC3s, DCs, macrophages and Tregs by finding that IL-1β production by macrophages induces GM-CSF production by ILC3s (Mortha et al., 2014). GM-CSF in the intestine is important for the maintenance of DCs and macrophages because when it
is absent, there is reduced RA production by DCs and insufficient conversion of naïve T cells into Tregs and thus an impairment of oral tolerance (Mortha et al., 2014).

RA in the intestine also influences ILC type, numbers and cytokine production. For instance, when maternal retinoids are low, progeny have reduced SLOs due to reduced lymphoid tissue inducers cells, a subet of ILC3s, which have been shown to induce SLO formation (Scandella et al., 2008; van de Pavert et al., 2014). Once progeny become adult mice, they have impaired clearance of virus due to small SLOs (van de Pavert et al., 2014). In adult mice, deficiency of Vitamin A in the diet results in impaired ILC3s and reduced IL-22 and IL-17A production by these ILCs (Spencer et al., 2014). This reduction culminates in impaired clearance of *Citrobacter rodentium*. While a diet lacking in Vitamin A results in lower ILC3s, it creates a skew towards ILC2s and upon helminth infection, and enhanced immunity to these worms (Spencer et al., 2014). In addition RA can also induce IL-22 production by ILCs and protect against chemically induced colitis (Mielke et al., 2013).

Cytokines secreted by ILCs in the intestine must be tightly regulated otherwise there is a susceptibility to IBD. In several models of colitis, the cytokine IL-23 can have both a pro- and anti-inflammatory role on colitis progression depending on the type of ILC cytokine production. In a model induced by CD40 antibodies that acts on DCs, IL-23 enhanced IFNγ and IL-17A production by Lineage^Thy1.2^+ ILCs in the intestine (Buonocore et al., 2010). Following Thy1.2 depletion, colitis was ameliorated and there was reduced pro-inflammatory cytokine production by ILCs in the gut (Buonocore et al., 2010). Another group using the same anti-CD40 model of colitis showed that IL-23 receptor signaling led to enhanced IL-22 production and increased colitis severity due to IL-22 dependent recruitment of pro-inflammatory neutrophils (Eken et al., 2014). Conversely, in DSS colitis models, IL-22 production by ILCs has been shown to be
protective by inducing several antimicrobial peptides from intestinal epithelial cells such as Reg3γ and β-defensins (Bishop et al., 2014; Cox et al., 2012). These peptides are thought to act to control over-proliferative micro-organisms (Zheng et al., 2008). IL-22 also acts to maintain the barrier integrity and through this mechanism protects upon inflammation induced by DSS (Zenewicz and Flavell, 2011). Clearly, the cytokines IL-23 and IL-22 can communicate with different cell-types depending on the inflammatory situation and determining what and how a particular immune communication pathway is triggered is important in discovering and modifying new and current treatments for IBD.

1.2.6 The Role of T Cells in the Intestine

T cells in the intestine are key players in the maintenance of homeostasis but can contribute to and are shown to accumulate greatly upon IBD. At both effector sites of the intestine, the intraepithelial (IEL) compartment and the LP, there are significant numbers and proportions of T cells present even in steady-state (Shale et al., 2013). At these effector sites, the majority of CD4+ T cells have an effector memory phenotype of high CD44 expression and low CD62 expression, suggesting that they are antigen experienced (Targan et al., 1995). Within the IEL compartment, the majority of T cells express CD8, either the conventional αβ heterodimer or the αα homodimer and of these, the majority expresses the γδ TCR not the αβ TCR (Cheroutre et al., 2011). CD4+ T cells are also present in the IEL compartment, the majority of which are αβ TCR expressing; however they are more prominent within the colon IEL compartment. In the LP, CD4+ αβ T cells are the greater proportion of T cells (Beagley et al., 1995).
1.2.6.1 Th17 Cells

Surprisingly, in steady-state, there is large portion of Th17 cells in the gut (Ivanov et al., 2006). Th17 cells secrete various cytokines such as IL-22, GM-CSF and IL17A in steady state and inflammation. While IL-17A, like IL-22, has been shown to recruit neutrophils into the intestine, the transfer of IL-17A deficient T cells into RAGKO mice exacerbates the systemic wasting disease in these mice, suggesting that IL-17A may act to repress IFNγ production (Eken et al., 2014; Miyamoto et al., 2003; O'Connor et al., 2009). The role of Th17 cells in the intestine upon steady-state is likely to contain any fungal or bacterial infections (Ivanov et al., 2009). Th17 cells express the transcription factor, RORγt and are induced and maintained by a variety of cytokines including, IL-21, IL-23, IL-1β, TGFβ and IL-6 (Ivanov et al., 2006; Izcue et al., 2008; Korn et al., 2007; Li et al., 2007; Shaw et al., 2012; Zhou et al., 2007). There are, however, conflicting reports on the importance of all these cytokines to induce and maintain Th17 cells; for instance, while it has been shown that mice deficient in TGFβ1 are impaired in their generation of Th17 cells, another report has suggested that Th17 cells can be generated from precursors with IL-6 and IL-23, and in combination with IL-1β, are sufficient to cause pathology in a model of experimental allergic encephalomyelitis (Ghoreschi et al., 2010).

Upon inflammation, while there are controversies to the purely pathogenic role of IL-17A, several studies have shown that IL-17A producing T cells enhance inflammation in the gut. Much of the pathogenic function of Th17 cells has been tied to the cytokine, IL-23. While IL-23 is not thought to purely drive Th17 development, it can maintain and modulate Th17 effector function (Ahern et al., 2010; McGeachy et al., 2009; Stritesky et al., 2008). Employing the T cell colitis model, Ahern et al. showed that the transfer of IL-23 receptor deficient T cells into RAG deficient mice was critical for the proliferation, accumulation and production of IL-17A and
IFNγ by Th17 cells (Ahern et al., 2010). Interestingly, the pathology of disease was only restricted to the intestine. In chemically induced colitis models, the pathogenicity of Th17 cells has also yielded conflicting results. Neutralization of IL-17A enhanced DSS-induced colitis in mice and DSS colitis in IL-17A deficient mice only resulted in a slight enhancement of disease with increased CD4+ effector T cells and CD11b-positive granulocytes-monocytes in the colon of these mice compared to wildtype mice (Ito et al., 2008; Ogawa et al., 2004).

1.2.6.2 Th1 Cells

While there are conflicting reports on the role of Th17 upon inflammation in the gut, the role of Th1 effector cells is clearer. Th1 cells are not prevalent in the intestine at steady state (Shale et al., 2013). Alternatively, upon inflammation and in particular with the induction of colitis by the transfer of CD45RB^{high} T cells into RAG deficient mice, the majority of the T cells in the gut of these mice secrete IFNγ (Strober and Fuss, 2011). The transcription factors, signal transducer and activator of transcription 1 (STAT1) and STAT4 are activated by IFNγ and IL-12 respectively (Zhu et al., 2010). This ultimately leads to the induction of T-bet, a transcription factor needed for the development of Th1 cells (Zhu et al., 2010). T-bet acts to drive the expression of IFNγ and the receptor IL-12Rβ2 to make Th1 cells more sensitive to IL-12 (Zhu et al., 2012). IL-12 along with IFNα and IL-27 can also induce T-bet induction and promote the differentiation of Th1 cells (Hibbert et al., 2003; Neurath, 2008; Villarino et al., 2008). In patients with Crohn’s disease, increased Th1 cells are characteristic of the intestinal inflammation, however treatment of the T cell transfer colitis in mice with anti-IFNγ antibodies or with patients with established Crohn’s disease proved to be ineffective in both scenarios (Okazawa et al., 2002; Reinisch et al., 2006).
1.2.6.3  Tregs

Another distinctive CD4+ T cell subset are Tregs. There are both Foxp3+ Tregs and CD4+ T cells that do not express Foxp3 but do secrete copious amounts of IL-10 called Tr1 cells (Maynard et al., 2007). Tr1 cells are in the small intestinal LP (Kamanaka et al., 2006). The intestinal Foxp3+ T cells consist both of the natural Tregs, which develop in the thymus, and inducible Tregs that are not derived in the thymus and induced likely by intestinal APCs that are specialized with the ability to secrete RA and TGFβ (Chaudhry et al., 2011; Coombes et al., 2007; Sakaguchi, 2004). The importance of CD4+ T cell production of IL-10 is evident in IL-10 deficient mice that spontaneously develop intestinal disease in specific pathogen free animal units (Kuhn et al., 1993). Development of colitis in these mice is dependent on the presence of CD4+ cells since colitis does not occur in RAG deficient mice or in IL-10 deficient mice depleted for CD4+ cells (Davidson et al., 1996). The cells that IL-10 acts on are evident by the expression of the receptor for IL-10, which is expressed on Th1, Th17 cells and APCs in the gut (Moore et al., 2001).

While all three of these CD4+ helper T cell subsets have seemingly distinctive function and phenotype in the intestine, there is evidence for plasticity between the T cells with subsets taking on the transcription factors or producing cytokines that are normally produced by one particular subset (Brucklacher-Waldert et al., 2014). For instance, CD4+ T cells that express both RORγt and T-bet and produce IL-17A and IFNγ have been found (Annunziato et al., 2007). These double-producing cells of IL17A and IFNγ are thought to promote disease pathogenesis in Crohn’s disease patients. Interestingly in human patients with IBD, Foxp3+Th17+ have also been identified, which are thought to be Foxp3+ Tregs that acquire the potential to secrete IL-17.
(Hovhannisyan et al., 2011). The plasticity among the CD4+ T cells subsets is likely due to the complex and intense cytokine milieu present in inflammation of the intestine with DCs, macrophages and ILCs all contributing to the severity of disease.

1.2.6.4 Homing of Intestinal T Cells

A common feature among T cell subsets is that they have similar induction of gut homing molecules in steady state and upon inflammation (Agace, 2008). Induction of gut homing molecules on T cells occurs at the local lymph nodes, typically the mLNs (Agace, 2008). Once homing molecules are expressed they then home to the LP or IEL effector compartment. RA has been shown to induce α4β7 and CCR9 on Tregs in steady-state and on effector T cells upon inflammation (Coombes et al., 2007; Hall et al., 2011). As a result, T cells home towards their respective ligands, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCL25 (Agace, 2008; Ando et al., 2005). Much of the evidence for intestinal T cell homing has been done in the small intestine. CCL25 is not expressed in the large intestine while MAdCAM-1 is, suggesting that the interaction of α4β7 with MAdCAM-1 is more important in the large intestinal T cell homing (Ando et al., 2005; Campbell and Butcher, 2002). Recently, it has been shown in two reports that the chemoattractant receptor, GPR15 is expressed on both T effector cells and Tregs and promotes their trafficking and recruitment to the large intestine (Kim et al., 2013; Nguyen et al., 2015). How GPR15 is induced and regulated is still not well understood. Homing molecule expression and regulation on T cells and on effector T cells in particular are of importance because they are targets for potential therapies for the treatment of IBD. While an antibody against α4, natalizumab has proved effective in treating patients with moderate to severe Crohn’s disease, it also has unanticipated neurological side effects (McLean et al., 2012).
It has been hypothesized that the severe side effects seen with natalizumab treatment is due to it blocking both α4β1 as well as α4β7 (McLean et al., 2012). Unlike natalizumab, vedolizumab specifically targets α4β7 and does not inhibit binding at VCAM-1 through α4β1 (Soler et al., 2009). In addition, therapeutics targeted toward other homing molecules such as CCR9 and MAdCAM-1 are also being developed (Lobaton et al., 2014). It is therefore of keen interest to understand how these homing molecules are regulated and to discover and understand how large intestinal T cell homing occurs.

1.3 Inflammatory Bowel Disease

IBD can be classified into two forms, ulcerative colitis and Crohn’s disease (Abraham and Cho, 2009). They are diseases of the intestinal tract and of the underlying mucosa. They are also generally chronic, remitting and their etiology is not known (Kaser et al., 2010). Ulcerative colitis is usually restricted to the large intestine and inflammation is continuous (Abraham and Cho, 2009). Crohn’s disease can occur at any part of the gastrointestinal tract but it generally occurs in the ileum and colon of patients and inflammation is transmural whereas the inflammation in ulcerative colitis occurs primarily in the mucosa (Abraham and Cho, 2009). IBD is thought to occur in genetically susceptible individuals where there is an inappropriate inflammatory response in the gastrointestinal system in the absence of a pathogen. Canada has one of the highest incidences of IBD and as of 2012, there are approximately 233,000 Canadians diagnosed with IBD, which corresponds to a prevalence of 0.67% of the population (Rocchi et al., 2012). In addition approximately 10, 200 new cases occur annually. While IBD is a debilitating disease to the individual affected by it, it is also a burden on society with annual costs over $1 billion dollars due to the costs of medications and hospitalizations (Rocchi et al.,
It is therefore necessary to find not only more effective therapeutics to treat disease but ultimately find a cure. Basic research using animal models of IBD have proved effective in directing the development of new drugs to treat IBD in patients and will likely also be important in understanding the etiology of disease.

### 1.3.1 Animal Models of Colitis: The Dextran Sodium Sulphate Model of Acute Colitis

The DSS model of colitis is widely used due to its simplicity and controllability. It also resembles aspects of human ulcerative colitis and drugs that are used widely to treat human IBD such as cyclosporine A, methotrexate and anti-IL12p40 antibodies are efficacious in the murine model of DSS colitis (Melgar et al., 2008; Okayasu et al., 1990). DSS is a water-soluble negatively charged sulfated polysaccharide and the molecular masses between 40-50 kDa result in colitis in mice (Chassaing et al., 2014). It is thought that DSS is toxic to colonic epithelial cells and through this toxicity, it creates permeability in the barrier and an influx of luminal bacteria into the LP resulting in inflammation (Perse and Cerar, 2012). Adjusting the percentage of it in the drinking water can alter the severity of DSS colitis and acute and chronic remitting diseases can also be induced. Furthermore, a model of colorectal cancer can be induced with DSS along with the administration of the carcinogen, azoxymethane (Tanaka et al., 2003). Interestingly, the use of the DSS colitis model to study the adaptive immune system is underused due to the observation that DSS colitis can be induced in mice without an adaptive immune system. However, recent studies have shown that lymphocytes can contribute to disease. When Kim et al. compared RAG1KO and C57Bl/6 mice on high dose (5%) and low dose (1.5%) DSS, weight loss and intestinal pathology was comparable in the high dose DSS experiment but the RAG1KO mice have lower systemic and local inflammation in the low dose experiment (Kim et
al., 2006). This indicates that at a lower dose of DSS, T or B cells can contribute to the inflammation induced by DSS. Morgan et al. take these results further by showing that antigen-specific T cells to an oral peptide can be detected systemically upon DSS colitis development, suggesting that T cells are activated specifically and can contribute to disease progression (Morgan et al., 2013).

1.3.2 Animal Models of Colitis: The CD45RB\textsuperscript{high} T Cell Transfer Model

The T cell transfer model of colitis has been key in identifying the various roles of T cells and their cytokines such as IFN\(\gamma\) and IL-17A in the pathogenesis of IBD (Groux et al., 1997; O'Connor et al., 2009; Powrie et al., 1993). In addition to effector Th1 and Th17 cells, Tr1 cells, Tregs and their cytokines such as IL-10 and TGF\(\beta\) have shown to be protective in this model (Groux et al., 1997; Li et al., 2007). Furthermore, intestinal myeloid cells and their cytokines such as IL-23 have also been implicated for their roles if perpetuating and regulating inflammation by inducing effector T cells (Ahern et al., 2008). It is thought that in the absence of regulatory T cells, adoptively transferred naive T cells into immunodeficient hosts recognize commensal antigens become activated and proliferate unheeded driving intestinal inflammation (Powrie and Coffman, 1993). The T cell transfer model mimics aspects of both Crohn’s disease and UC since upon colitis induction, inflammation is skewed towards a Th1 response like Crohn’s disease but the inflammation is continuous like UC (Strober and Fuss, 2011).

1.3.3 Extraintestinal Complications of IBD

The development of IBD in an individual is not only restricted to inflammation in the intestine but also results in other various extraintestinal complications such as weight loss and
anemia (Danese and Fiocchi, 2011). As many as one-third of IBD patients develop anemia, which may result from either iron deficiency or from ineffective erythropoiesis and shortened red blood cell survival, also known as anemia of chronic diseases (ACD) (Gasche et al., 2004). ACD is mediated by excessive production of TNFα, IL-1 and IL-6 and can be treated with exogenous erythropoietin (EPO) (Nairz et al., 2011). EPO is a hormone produced by the kidney and/or liver that acts on committed erythroid progenitors in the bone marrow to stimulate RBC production (Koury et al., 1991). Interestingly in mice induced with colitis, serum EPO is not always decreased (Carter et al., 2013). Anemia may instead result from unresponsive bone marrow progenitors to EPO leading to inefficient red blood cell (RBC) generation. Decreased EPO levels or inefficient EPO-receptor responsiveness on erythroid progenitors can be overcome, however, by the addition of exogenous EPO (Nairz et al., 2011). Exogenous EPO decreases expression of pro-inflammatory mediators such as IL-12, IL-6 and TNFα in a model of TNBS colitis and decreases the development of DSS colitis in EPO-receptor deficient mice (Nairz et al., 2011). The development of anemia from such chronic diseases like IBD could be considered a secondary consequence of disease, but intriguingly by treating anemia with EPO, local gut and systemic inflammation in mouse models of colitis is alleviated. Thus, treatment with EPO targeting the generation of erythrocytes may be also provide an unanticipated alternate therapy for IBD treatment (Nairz et al., 2011).

1.4 Erythropoiesis

Erythropoiesis and hematopoiesis are tightly regulated multi-step processes to generate mature erythrocytes and immune cells. To generate mature erythrocytes, the process begins in the bone marrow at the HSC stage and diverges at the multipotent common myeloid progenitor
CMPs can differentiate into megakaryocyte-erythrocyte progenitors (MEP) or the granulocyte-macrophage progenitor (GMP) (Akashi et al., 2000). GMPs eventually go on to become monocytes or granulocytes (Akashi et al., 2000). MEPs differentiate into more mature burst-forming units-erythroid (BFU-E) and then into colony-forming units-erythroid (CFU-E) (Buck et al., 2009). BFU-E and CFU-E are the earliest progenitors of the erythroid lineage pathway that are identifiable in culture (Gregory and Eaves, 1977). IL-3 and stem cell factor (SCF) can act on BFU-E and CFU-E in culture to expand their numbers. It is also at the BFU-E stage where the EPO receptor is expressed (Buck et al., 2009). EPO can control the survival, proliferation and differentiation of erythroid progenitors by activating a number of signaling pathways including the phosphatidylinositol 3 kinase (PI3K), the Janus kinase (JAK)/STAT and the mitogen-activated protein kinase (MAPK)/extracellular signal-related kinase (ERK) pathways (Hodges et al., 2007; Zhao et al., 2006). The survival and terminal differentiation of erythroid precursors into erythroblasts and finally erythrocytes is based on the interaction of EPO and its receptor and also on the expression of transcription factors such as GATA-1 and ELKF (Gregory et al., 1996). Erythroid progenitors develop in the bone marrow, blood and spleen. The different stages of maturity can be differentiated by their expression of CD71 (the transferrin receptor) and TER119 (Shimizu et al., 2008). The less mature erythroblasts, also known as pro-erythroblasts express CD71 only and as they mature they gain surface TER119 are double positive, then begin to lose CD71 expression (Koulnis et al., 2011). The cells then become smaller and lose the nucleus, as they become mature erythrocytes. When cells are at their final stages of erythrocyte maturity and as fully formed erythrocytes, they only express TER119 (Koulnis et al., 2011).
Interestingly, recently it has been shown that CD71+ erythroblasts and EPO can be directly suppressive on pro-inflammatory cells of the immune system. Nairz et al. show that in a mouse model of colitis, exogenous EPO can directly suppress pro-inflammatory cytokine production by myeloid cells expressing the EPO receptor and ameliorate colitis (Nairz et al., 2011). A study by Elahi et al. recently showed that neonates have increased TER119+CD71+ cells that function to suppress the production of pro-inflammatory cytokines by neonatal intestinal myeloid cells (Elahi et al., 2013). The suppressive nature of TER119+CD71+ erythroblasts and EPO can also be detrimental to the host in bacterial infection. The study by Nairz et al. demonstrated that while exogenous EPO was beneficial upon colitis by alleviating inflammation, excessive EPO also leaves the host susceptible to bacterial infection due to reduction of TNFα. (Nairz et al., 2011) Furthermore, a study by Jackson et al. showed that the splenomegaly that occurs in systemic Salmonella infection in mice is associated with increased TER119+CD71+ erythroblasts in the spleen (Jackson et al., 2010). Neutralization of EPO reduces the TER119+CD71+ population and provided some control of the infection in the host (Jackson et al., 2010). Interestingly, the tyrosine phosphatase, CD45 is expressed on erythroid progenitors, but the percentage of CD45+ cells decreases as erythroblasts become more mature and CD45 is not expressed on erythrocytes (Craig et al., 1994; Lansdorp et al., 1990).

1.5 CD45

1.5.1 CD45 Structure and Substrates

CD45 is a leukocyte specific molecule expressed from the HSC to all mature white blood cells but as red blood cells mature, the percentage of CD45 decreases (Craig et al., 1994; Lansdorp et al., 1990). It is a transmembrane, protein tyrosine phosphatase and has the potential
to regulate and influence blood cell development and immune function (Hermiston et al., 2003). Indeed the generation of CD45 deficient mice has shown a clear role for CD45 in lymphoid development and function and some work has shown that CD45 can influence the JAK/STAT signaling pathway, which are often downstream of cytokine receptor signaling (Irie-Sasaki et al., 2001; Saunders and Johnson, 2010). Given the role of cytokines in regulating intestinal inflammation and feedback mechanisms in development, it was of interest to determine if CD45 played a role in regulating intestinal immune responses.

In the extracellular portion, CD45 is heavily glycosylated, cysteine rich and has three fibronectin type-III domains. The intracellular portion of CD45 consists of two tyrosine phosphatase domains. The membrane proximal phosphatase domain is the catalytic part of CD45. CD45 can also exist in several alternatively spliced isoforms of the extracellular domain resulting in splicing from exons 4, 5 and 6 (Figure 1.2).

The best known substrates for CD45 are the Src family kinases (SFKs). SFKs include Lck, Fyn, Lyn and Hck (Saunders and Johnson, 2010). Lck in T cells and Lyn in B cells have been well characterized. SFKs have both a negative and positive regulatory site that can be dephosphorylated by CD45. Phosphorylation at the negative regulatory phosphorylation site causes an intramolecular interaction that creates an inactive conformation (Hermiston et al., 2003). When CD45 dephosphorylates this negative regulatory site such as Y505 in Lck, it creates a ‘primed’ SFK. SFKs can then cluster and transphosphorylate themselves to become active. CD45 can also dephosphorylate SFKs at the positive regulatory site such as Y394 in the activation loop of Lck, downregulating its activity and returning the SFK to its ‘primed state’ (Hermiston et al., 2003) (Figure 1.3). The JAKs have also been implicated as substrates for CD45. In CD45 deficient T cells, B cells and mast cells, JAK kinases are hyperphosphorylated in
response to a number of cytokine stimulations including IL-3, IL-4 and IFNα (Irie-Sasaki et al., 2001). Notably, because SFKs can regulate a number of immunomodulatory proteins involved both in antigen receptor signaling and cytokine signaling, JAK kinases may also be regulated as a result of the CD45 regulation of SFKs.

1.5.1 The Roles of CD45 in the Adaptive Immune System

CD45 is well established as an SFK phosphatase in antigen receptor signaling in T and B lymphocytes. Three CD45 knockout mouse lines have been generated targeting exon 6 (CD45E6KO), exon 9 (CD45E9KO) and exon 12 (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999). These CD45 deficient mice have revealed a significant role for CD45 in T cell development and function. While both CD45E6KO and CD45E9KO mice have T cells in the spleen, the numbers are only 5-10% that of wildtype mice (Byth et al., 1996; Kishihara et al., 1993). This is thought to be due to a defect in thymocyte development in CD45 deficient mice where there is a marked reduction in the transition of CD4+CD8+ double positive thymocytes to single positive thymocytes (Byth et al., 1996; Kishihara et al., 1993). Analysis of CD45 deficient thymocytes reveals that CD45 positively regulates TCR signalling by promoting the activation of SFKs since Lck and Fyn are hyperphosphorylated in CD45 thymocytes (Stone et al., 1997).
**Figure 1.2 CD45** (Johnson et al., 2012). CD45 is a glycosylated transmembrane protein phosphatase with three extracellular alternatively spliced regions, a cysteine region and fibronectin type III-like repeats. The intracellular domain of CD45 contains two phosphatase domains, of which D1 is catalytically active.
Figure 1.3 CD45 dephosphorylates the SFK, Lck, to create a primed state (Johnson et al., 2012). CD45 dephosphorylates the negative regulatory site, Y505 on Lck to create a primed state. On active Lck, CD45 dephosphorylates the positive regulatory site, Y394 on Lck to return it back to the primed state.
In addition, basal and TCR-stimulated Zap70 recruitment is reduced as is CD3ζ and CD3ε phosphorylation (Stone et al., 1997). Furthermore, CD45E6KO mice have a subset of peripheral T cells that express the isoform of CD45RB, while CD45E9KO mice have no expression of CD45 on lymphocytes (Kong et al., 1995). The expression of CD45RB on the T cells in CD45E6KO mice is lower than normal and there is also an abnormal regulation of expression after in vitro stimulation (Kong et al., 1995). This suggests that the CD45 sufficient T cells are not appropriately functional as the level of expression of CD45 is associated with its function (Zikherman et al., 2012). However, CD45E6KO T cells abnormally express LFA-1 and CD44, exhibiting a more activated phenotype (Kong et al., 1995). These phenotypically activated CD45+ T cells within the CD45E6KO mice may also be functionally activated but this has not been well explored in vivo. Furthermore, a previous study has shown that when the diet of CD45E9KO mice are altered by supplementation with human infant formula, CD45E9KO mice had increased intracellular IFNγ, IL-2 and TNFα from αβ T cells and γδ T cells from the small intestine (Lopez and Holmes, 2004).

In addition to its role in T cell development, CD45 has also been implicated in regulating T cell adhesion. CD45 deficient T cell lines have an enhanced ability to adhere to fibronectin via the integrin α5β1 (VLA5), but not α4β1 (VLA4) (Shenoi et al., 1999). CD45 deficient T cell lines were shown to have enhanced spreading when interacting with immobilized CD44 antibodies and this interaction correlated with induction of tyrosine phosphorylation of focal adhesion family kinases, Pyk2 and FAK (Li et al., 2001). Subsequently, the elongated cell spreading observed in CD45 deficient T cell lines was shown to be consistent with enhanced Lck Y394 phosphorylation in these same T cell lines as well as CD45 deficient ex vivo thymocytes (Wong et al., 2008). While this work has highlighted an important role for CD45 in T cell
spreading and adhesion, the consequences of this in vivo are still not understood.

CD45 in B cells also affects SFK signalling but does not completely block their activated signals (Fleming et al., 2004). CD45 deficient mice have more muted B cell development defects than T cells and peripheral B cell numbers are in fact increased, though there are alterations in B cell subsets (Justement et al., 1991).

1.5.2 The Roles of CD45 in the Innate Immune System

Unlike in T and B cells, the role of CD45 in innate immune cells is less comprehensively studied. Much of the work on various innate immune cells has been done in vitro. These studies done on natural killer (NK) cells, mast cells, macrophages and DCs do however give insights into the role of CD45 as a regulator of cytokine production and adhesion. CD45 deficient NK cells from CD45E6KO have deficient cytokine and chemokine production in response to ligands to NK1.1, Ly49D and NKG2D (Hesslein et al., 2006). Bone marrow derived mast cells also have reduced pro-inflammatory cytokine production as well as reduced IgE mediated degranulation (Grochowy et al., 2009). These results show that CD45 in NK cells and mast cells positively regulates pro-inflammatory cytokine production. Alternatively, in bone marrow derived CD45 deficient DCs (BMDC), CD45 has been shown to play both a positive and negative role in pro-inflammatory cytokine production (Cross et al., 2008; Piercy et al., 2006). CD45 deficient BMDC, that have been grown in culture and differentiated with GM-CSF, have increased production of the pro-inflammatory cytokines, IL-12, IL-6 and TNFα in response to TLR-2 and TLR-9 ligands but only a slight effect to the TLR-4 ligand, LPS (Cross et al., 2008). Conversely, in response to a TLR-3 ligand, CD45 deficient BMDCs have reduced IFNβ production (Cross et al., 2008). Moreover, CD45 deficient mice are less efficient at secreting IFNα in response to
lymphocytic choriomeningitis virus infection and were unable to clear the infection (Montoya et al., 2006). These results suggest that CD45 negatively affects MyD88 dependent TLR signaling that leads to pro-inflammatory cytokine production but positively affects MyD88-independent, TRIF-dependent signaling that leads to type I interferon production. Like T cells, CD45 deficient bone marrow derived macrophages (BMDMs) have aberrant adhesion properties. In both GM-CSF and M-CSF derived CD45 deficient BMDMs, have reduced spreading and stretching and less movement in culture (Roach et al., 1997; St-Pierre and Ostergaard, 2013).

1.5.3 Summary

Taken together, the altered cytokine production and adhesion properties in CD45 deficient innate immune cells reveals the multiple roles CD45 plays in regulating immune cell function. Nevertheless, there are many aspects of CD45 biology that are still unanswered. For instance, the consequences of enhanced pro-inflammatory cytokine production by CD45 deficient DCs and altered adhesion properties in both T cells and macrophages have not been explored in vivo or upon disease. Furthermore, the function of CD45 on ILCs has not been analyzed at all. Because CD45 has been shown to regulate cytokine production, adhesion and antigen receptor signaling, in different cell-types, I hypothesize that it could play a role in cell-to-cell communication in vivo. Understanding how CD45 functions in vivo in health and disease may reveal how adhesion and pro-inflammatory cytokine function affect disease and potentially uncover other avenues of disease treatment.

1.6 Research Objective

CD45 is expressed on all blood cells except for mature erythrocytes and is well
characterized for regulating signalling pathways affecting various cell functions such as cytokine production, cell adhesion, motility and cell development. The establishment of CD45 knockout mice has revealed that CD45 is required for T cell development and that it also regulates T and B cell antigen receptor signalling. Interestingly, it has been shown that there is a point mutation in the gene, *PTPRC*, encoding CD45 is associated with the development of multiple sclerosis in some families (Jacobsen et al., 2000). However, recently this same genetic polymorphism was not found to be associated in Swedish patients with myasthenia gravis, a neuromuscular disorder similar to multiple sclerosis (Ramanujam et al., 2010). This conflicting data suggests that CD45 may play a role in the development and/or progression of autoimmune diseases but to elucidate these roles systematically and especially in the adaptive immune system is complex due to the low T cell numbers in the CD45KO mice. Since many diseases are T cell mediated and CD45KO T cells are thought to be non-functional, it is unsurprising that there is little known about the roles of CD45 in vivo and if one or many of its various roles shown in vitro are recapitulated in vivo. Furthermore, many of the in vitro results of enhanced cytokine production by CD45 deficient innate immune cells have not been evaluated in vivo in steady-state or in inflammation.

Interestingly, the evaluation of low T cell numbers in the periphery of CD45KO mice has only been done in the spleen and lymph nodes (Byth et al., 1996; Kishihara et al., 1993). CD45 deficient T cells in other peripheral tissues such as the intestine have not been well characterized in steady state and not at all upon inflammation. It is of importance to characterize the role of CD45 on immune cells in the intestinal immune system to determine if CD45 can regulate pro-inflammatory cytokine production or adhesion of immune cells affecting diseases such as colitis thereby revealing new avenues of treatment.

Based on the already established roles of CD45 in the immune system, the major
The objective of this project is to determine the roles of CD45 in intestinal inflammation. CD45 is likely to effect cytokine signalling in the intestine and may have a role in immune cell communication. CD45 has been shown to regulate cytokine production by TLR activation and therefore may also influence intestinal innate immune responses. Furthermore, CD45 has been shown to regulate integrin and adhesion molecules in vitro and therefore may regulate homing or retention of intestinal immune cells. Finally, CD45 has clear roles in T cell and B cell activation but its role on these adaptive immune cells has not been established in inflammation in vivo. Taken what is known about CD45 in various immune cells of the innate and adaptive immune system, I hypothesize that CD45 on the adaptive immune system will result in lower effector T cells in the gut while CD45 on dendritic cells and other innate immune cells in the intestinal immune system will play a critical role in the pathogenesis of colitis by negatively regulating pathways important for cytokine production.

The first part of this project will be to determine the effect of CD45 deficient immune cells in the DSS model of colitis. Reconstituting DSS in the drinking water disrupts the epithelial barrier of the gut, which results in an influx of microbes into the LP (Chassaing et al., 2014; Okayasu et al., 1990). This model involves a variety of cell-types that contribute to intestinal inflammation and weight loss; since not only are DCs activated by the microbes but so are other innate immune cells such as macrophages and epithelial cells resulting in the activation of ILCs and T cells. The adaptive immune system also contributes to inflammation in the DSS model of colitis but colitis still occurs in RAGKO mice (Kim et al., 2006). I hypothesize that DSS colitis will be less severe in CD45 deficient mice because gut effector T cell frequency and number will be reduced.

In the second part of this project, I sought to determine the effect of CD45 deficient
myeloid cells in a T cell mediated model of inflammatory bowel disease. The T cell transfer model of colitis will assess the ability of CD45 deficient dendritic cells and macrophages to induce normal naïve T cells to become inflammatory. The T cell transfer model is conducted in mice that do not have regulatory T cells; therefore, when naïve T cells are adoptively transferred into these mice, these naïve T cells proliferate and expand into pathogenic Th1 and Th17 cells in the gut and associated lymphoid tissues (Powrie et al., 1993). I hypothesize that colitis will be more severe in the CD45 deficient mice in the T cell transfer model because CD45 deficient myeloid cells will be hyper-responsive to TLR ligands from the commensal flora and in the absence of regulatory T cells, will produce more pro-inflammatory cytokines and induce increased pathogenic Th17 and Th1 cells than in CD45 sufficient mice.
Chapter 2: Materials and Methods

2.1 Mice

C57BL/6, CD45KO Exon 9 knockout mice (Byth et al., 1996) and RAG1KO mice were obtained from The Jackson Laboratory. CD45 Exon 6 knockout mice (Kishihara et al., 1993) were obtained from Dr. J. Penninger and backcrossed for six generations onto the C57Bl/6 background. The CD45 Exon 9 deficient mice were backcrossed for at least nine generations onto the C57BL/6 background. C57BL/6, CD45KO Exon 9 and CD45 exon 6 mice were re-derived, bred and used in the Containment Level 1 of the Centre for Disease Modeling Animal Facility. CD45E9RAGKO and CD45E6RAGKO mice were generated by crossing CD45KO mice with RAG1KO mice to homozygocity. Mice were bred under specific pathogen free conditions at the Wesbrook Animal Unit and then transferred into pre-Containment Level 1 of the Centre for Disease Modelling Animal Facility at the University of British Columbia. All mice were also re-derived into Containment Level 1 of the Centre for Disease Modeling Animal Facility. The mice were maintained and used for experimentation in accordance with the Canadian Council of Animal Care Guidelines and with approval from the University of British Columbia Animal Care Committee. Mice were used between 6 and 14 weeks of age and matched for age and gender.

2.2 Induction of DSS Colitis, Cell Transfers and Injection of All-Trans Retinoic Acid and GM-CSF

Experimental colitis was induced by dissolving 3% (w/v) dextran sodium sulphate (molecular weight., 36,000-50,000 MP Biomedicals) into drinking water and given to mice for 7
days. Normal drinking water was then given for an additional 1-2 days. The animals were weighed daily and also assessed for clinical signs of colitis such as rectal bleeding. Polyclonal T cells were isolated from C57BL/6 spleens and lymph nodes (mesenteric, auxiliary and inguinal) by negative depletion after labelling cells with biotinylated antibodies, CD11c (N418), Ter119 (TER119), CD11b (M1/70) and B220(RA3-6B2) and then adding anti-biotin beads (Miltenyi) to pass through magnetic columns (Miltenyi) for collection of the unlabeled fraction. T cells were at least 93% pure and 3-5x10⁶ T cells/mouse were injected once intravenously on day 0 of a DSS experiment. The T cells included Tregs, CD4+ and CD8+ αβ T cells and γδ T cells. Where indicated, 200 µg/mouse of all-trans retinoic acid (Enzo Life Sciences), 5 µg/mouse recombinant mouse GM-CSF (BioLegend), PBS or vehicle, a mixture of 16% DMSO in peanut oil (Sigma) were injected intraperitoneally every other day from day 1 of a DSS experiment.

2.3 T Cell Transfer Colitis Induction

A single cell suspension was obtained by mechanical disruption of C57Bl/6 mice spleens between two frosted slides. The red blood cells were lysed with 0.84% ammonium chloride, 2mM Tris-HCl pH 7.2 buffer at room temperature for 5 minutes. The cells were then incubated with biotinylated antibodies for Ter119, B220, CD11b and CD8 for 20 minutes at 4°C, washed with a solution of 1XPBS, 0.5% BSA and 2mM EDTA and then incubated with anti-biotin antibodies (Miltenyi) for another 20 minutes at 4°C. The cells were put through a magnetic column (Miltenyi) and the negative fraction was collected and labelled with PECy7 conjugated anti-CD4, PE conjugated anti-CD25 and FITC anti-CD45RB (all from eBioscience) and sorted for the CD4+CD25-CD45RBhigh naïve T cells on a BD Aria™ or Influx™. 4x10⁵ naïve T cells
in 200ul sterile PBS were then injected i.p. into RAGKO and CD45RAGKO mice and development of disease was based on weight loss by RAGKO mice.

2.4 Histological Grading of Colon Inflammation

Mice were euthanized when physical symptoms of disease were evident in the RAGKO mice usually around 4 weeks at the Wesbrook Animal Unity and 2-3 weeks in pre-Containment Level 1 at the Centre for Disease Modeling. For colon sections, approximately 0.5cm of the distal colon was put in 10% formalin. 5 to 7 microns of paraffin-embedded sections were stained with hematoxylin and eosin by Wax-It Histology Services Inc. For assessment of disease severity, colon sections were graded blindly and semi-quantitatively based on inflammatory cell (lymphocyte) infiltration, epithelial cell hyperplasia, mucin depletion, transmural inflammation, and ulceration. A grade of 0 (no change), 1(intermediate), or 2 (severe) was assigned to each parameter and then summed to generate a final score/10 per section.

2.5 Cell Isolation

Single cell suspensions from mesenteric lymph nodes and spleens were isolated by incubating minced organs with 1 mg/ml of collagenase IV (Worthington) for 20 minutes and passing them through 70 µm strainers. For spleens, the red blood cells were lysed with 0.84% ammonium chloride, 2 mM Tris-HCl pH 7.2 buffer at room temperature for 5 minutes. For analysis of Ter119+ cells, the femurs and tibias were flushed and single cell suspensions of the mLNs and spleens were obtained by mechanical dissociation with two frosted glass slides without RBC lysis. Intestinal lamina propria cells were isolated from the large intestine. In brief, colons from the caecum to the rectum were dissected from euthanized mice and opened
longitudinally then cut into approximately 0.5 cm pieces. Colon pieces were then incubated for 20-30 minutes at 37°C in a solution of PBS, 5% FCS and 4mM EDTA for at least 3 washes to remove epithelial cells. Colon pieces were washed twice with a solution of PBS and 5% FCS to remove the EDTA and then minced with a scalpel, incubated twice at 37°C for 40 minutes in a solution of RPMI, 5% FCS and 1mg/ml of Collagenase IV and passed through a 70 µm strainer to obtain the lamina propria cells (Uhlig et al., 2006a).

2.6 Intracellular Staining and Flow Cytometry

Single cell suspensions were blocked for FcR binding with 2.4G2 cell culture supernatant then labelled direct fluorochrome conjugates for the surface markers CD11c (N418), CD11b (M1/70), CD103(2E7), MHCII (I-A/I-E), Gr1 (RB6-8C5), NK1.1(PK136), NKp46 (29A1.4), CCR9 (CW-1.2), α4β7 (DATk32), TCRβ (H57-597), CD8α (53-6.7), CCR7 (4B12), CD25 (PC61.5), CD71 (R17217), TER119, Sca1 (D7), CD34 (RAM34), cKit (2B8), CD16/32 (93), TCRβ (H57-597), and CD4 (GK1.5) from eBioscience. Thy1.2 (30-H12), CD19 (1D3), CD3 (2C11), and CD44 were from the Biomedical Research Centre Ab Facility. CD64 (X54-5/7.1) from BioLegend. Antibodies against lineage included a cocktail of the following: NK1.1 (PK136), CD4 (GK1.5), CD3 (17A2), CD19 (1D3), Ter119, CD11b (M1/70), CD11c (N418), Gr1 (RB6-8C5), CD8 (53-6.7), FcεRI (MAR-1). Isolated cells from the spleen and lamina propria were stimulated with PMA (50 ng/ml) and Ionomycin (500 ng/ml) for 4-6 hours in the presence of Brefeldin A (10 µg/ml), which was added at the same time, for the assessment of cytokine production. Intracellular labelling was performed with the Foxp3 fixation/permeabilization concentrate and associated buffers (eBioscience) for IL-22 (1H8PWSR), IFNγ (XMG1.2), IL-17A (ebio17B7), RORγt (B2D), Foxp3 (FJK-16s) and TNFα.
(MP6-XT22) from eBioscience and GM-CSF (MP1-22E9) from BioLegend. Samples were analyzed on a FACSCanto or LSR II (BD) with FlowJo software (Tree Star) with isotype controls to determine gating and exclude non-specific binding. The presence of aldehyde dehydrogenase activity in cells was determined using the ALDEFLUOR staining kit (STEMCELL Technologies) as per the manufacturer’s instructions. All cells were stained with a viability dye, either DAPI or 7-AAD for surface staining or with a fixable/viability dye (Invitrogen) for intracellular staining.

2.7 Cytokine Assays

IL-6, TNFα, and GM-CSF cytokine secretion was analyzed by ELISA or Flowcytomix (eBioscience) bead array according to manufacturer’s instructions. Spleen and colon explants were washed with 1XPBS with 5% FCS, weighed to 0.05g and cultured in serum free RPMI media supplemented with penicillin, streptomycin and gentamycin overnight at 37°C. Serum was obtained by cardiac puncture.

2.8 Statistical Analysis

Statistical analyses were performed using Graphpad Prism software (version 5.0). Data were analyzed using the unpaired students t test. For multiple analyses, ANOVA was used and where significance was found, the Tukey-Kramer multiple comparisons test was used for two-way and the Bonferroni test was used for one-way ANOVA for identifying differences between groups. When $P < 0.05$, the statistical difference was considered significant.
Chapter 3: CD45 Regulates GM-CSF, Retinoic Acid and T Cell Homing in Intestinal Inflammation

3.1 Introduction and Rationale

While both CD45E6KO and CD45E9KO mice have T cells in the spleen, the numbers are only 5-10% that of wildtype mice (Byth et al., 1996; Kishihara et al., 1993; Saunders et al., 2014). In addition, CD45E6KO mice have a subset of T cells that express a single isoform of CD45 (CD45RB), while CD45E9KO mice have no expression of CD45 on lymphocytes (Byth et al., 1996; Kishihara et al., 1993). CD45E6KO T cells express higher levels of LFA-1 and CD44, exhibiting a more activated phenotype (Kong et al., 1995), as do CD45E9KO T cells (A.S. unpublished observations). These phenotypically activated CD45+ T cells may also be functionally activated but this has not been explored in vivo. Furthermore, a previous study has shown that T cells in the large intestine of CD45E9KO mice had a more activated phenotype and could produce both Th1 and Th2 cytokines (Lopez and Holmes, 2005). Taken together, these results suggest that CD45 on T cells may limit their activation.

In addition to T cells, intestinal myeloid cells including DCs and macrophages are also important in maintaining homeostasis in the gut. In steady state, macrophages secrete IL-10 in order to maintain the regulatory T cell population, their own regulatory function and to generally counter any unwanted inflammatory responses (Denning et al., 2007; Rivollier et al., 2012; Zigmond et al., 2014). In steady state, dendritic cells (DC) are also key components of the intestinal immune system, where they can migrate to the mesenteric lymph nodes (mLN) via a CCR7-dependent mechanism to induce regulatory T cells (Tregs) (Annacker et al., 2005; Schulz
et al., 2009; Worbs et al., 2006). In addition, the CD103+ subset has been shown to secrete RA and TGFβ to promote Treg generation (Coombes et al., 2007; Sun et al., 2007). Intestinal DCs and macrophages also secrete cytokines in steady state and upon inflammation that influence the function of ILCs in the gut. Conversely, GM-CSF production by ILCs is important for maintaining intestinal myeloid population numbers; in the absence of GM-CSF, there is a reduction in DCs and macrophages in the intestine, which ultimately leads to lower RA production, and in a model of oral tolerance, insufficient generation of Tregs (Mortha et al., 2014). It is still unclear what the consequences are of GM-CSF production by ILCs upon inflammation. In addition to GM-CSF, it has been well characterized that ILCs secrete IL-22, IFNγ and IL17A (Buonocore et al., 2010; Satoh-Takayama et al., 2008; Takatori et al., 2009). IL-23 production by intestinal myeloid cells promotes the production of these cytokines by ILCs, but in addition to IL-23, TNF-like protein 1A (TL1A), RA and IL-1β production by myeloid cells also induce cytokine production by ILCs (Buonocore et al., 2010; Cox et al., 2012; Eken et al., 2014; Longman et al., 2014; Mielke et al., 2013; Mortha et al., 2014).

Given that CD45 deficient innate immune cells have been shown to be produce enhanced cytokines and since cytokines in the gut are critical mediators of communication, I wanted to characterize the role of CD45 on intestinal innate cells in addition to the role of CD45 on T cells. In this report, to do so, I characterize the CD45E6KO and the CD45E9KO mice in steady state and in DSS-induced inflammation and also cross these mice onto RAG1KO mice to characterize the CD45−/− innate immune system in the absence of CD45KO T cells. I describe a novel role for CD45 on T cells and on innate immune cells in intestinal inflammation, define the role of ILC GM-CSF production upon inflammation, provide an inflammatory role for RA in inducing
effector T cell homing and show that without CD45 RA production by CD103+ DCs and GM-CSF production by ILCs is compromised.

3.2 Results

3.2.1 The loss of CD45 on T Cells Increases Gut Homing T Cells and Promotes Inflammatory Cytokine Production

CD45−/− mice have a block in thymocyte development that results in only 5-10% of the normal T cells in the spleen and lymph nodes (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999). To investigate the role of CD45 in vivo and on gut effector T cells, we induced acute colitis in wildtype (WT), CD45E6KO and CD45E9KO mice by administering 3% DSS in the drinking water for 7 days and then normal drinking water for an additional 2 days. We hypothesized that colitis would be less severe in the CD45−/− mice because they would have insufficient effector T cells to exacerbate inflammation upon colitis development. Contrary to our hypothesis, CD45−/− mice were more susceptible to DSS colitis compared to WT mice (Figure 3.1A and Figure 3.1B). CD45E6KO mice had significantly more weight loss than WT mice on DSS and had lost 20% of their original weight by day 9 (Figure 3.1A). CD45E6KO also had significantly shorter colons compared to WT mice on day 9 indicative of more severe intestinal inflammation (Figure 3.1B). In addition, CD45E9KO mice had shorter colons than WT mice on day 9 of DSS colitis indicative of enhanced gut inflammation in the CD45−/− mice compared to WT mice (Figure 3.1B). Prior to colitis, CD45E6KO mice had increased total LP cell number compared to WT mice, while CD45E9KO mice had increased splenic cell numbers.
Figure 3.1. CD45KO mice develop more severe DSS-induced colitis. (A) Mice were given 3% (w/v) DSS in their drinking water for 0-7 days and then put on water for an additional 2 days. Representative experiment is shown of % loss of body weight, repeated 7-10 times with at least 3-5 mice per group. Students t test was used for comparison of CD45E6KO or CD45E9KO to WT mice. (B) Colon length of control mice and of mice on day 9 of DSS colitis. (C) Total cell number and T cell frequency and number in the spleen of control and on day 9, post colitis of WT, CD45E6KO and CD45E9KO mice. Data is pooled from at least two experiments, n=6-27 mice/genotype. (D) Total cell number and T cell frequency and number in the mLNs of control mice and of mice on day 9 post colitis. Data is pooled from at least two experiments, n=6-15 mice/genotype. (E) Total cell number and T cell frequency and number in the colon LP of control mice and of mice on day 9, post colitis. Data is pooled from at least two experiments, n=6-24 mice/genotype. Data represent mean ± SD. P values were calculated with ANOVA; *p<0.05, **p<0.01, ***p<0.001.
(Figure 3.1C-E). On day 9-post colitis, there was no difference in the cell number in the spleen, mesenteric lymph nodes (mLNs) or LP between WT, CD45E6KO and CD45E9KO mice (Figure 3.1C-E). As previously reported, control CD45E6KO and CD45E9KO mice had significantly reduced T cells in the spleen and mLNs compared to WT mice prior to colitis (Figure 3.1C and Figure 3.1D). In addition, control CD45−/− mice had reduced T cell frequency and number in the LP as in the periphery compared to WT mice (Figure 3.1E). Post DSS colitis, the reduction of CD45−/− T cells in the spleen and mLNs compared to WT mice was maintained (Figure 3.1C and 3.1D). Unexpectedly, there was no longer any difference in the T cell frequency or number in the colon of CD45E6KO mice compared to WT mice, while there was still an increase in T cells in the LP of CD45E9KO mice, the frequency and numbers of T cells were still significantly lower by about two-fold in the CD45E9KO mice compared to WT mice (Figure 3.1E).

To determine why there might be an equal or only a two fold difference in T cell numbers in the LP after DSS colitis in the CD45KO mice when they still had significantly reduced numbers of T cells in the spleen and mLNs, we looked at the expression of the gut-homing molecule, α4β7 (Gorfu et al., 2009; Wagner et al., 1996), as the loss of CD45 had previously been implicated in enhancing CD44- and integrin-mediated adhesion events (Shenoi et al., 1999; Wong et al., 2008). We assessed the expression of the adhesion molecule α4β7 on T cells in the mLNs post colitis and found α4β7 expression on CD45−/− T cells to be significantly increased (Figure 3.2A and 3.2B). This suggested that the enhanced expression of the homing molecule α4β7 on CD45−/− T cells may help account for the increased presence of CD45−/− T cells in the gut post colitis. The reduced numbers seen in the gut of CD45E9KO mice may be a reflection of a more severe reduction in total T cells in the CD45E9KO mice as the percentage of α4β7+ T cells
Figure 3.2. CD45KO mice have comparable gut homing T cells and enhanced IFNγ production by colon T cells post DSS. (A) Representative flow cytometry labeling of the integrin α4β7 on CD4+ and CD8+ T cells in the mLN on day 9 post DSS colitis. (B) Graphs of pooled data from two experiments of the frequency, cell number and MFI of α4β7+ CD4+ CD8+ T cells, n=6 mice/genotype. Data represent mean ± SD and ANOVA was used to calculate P values. (C) Representative flow cytometry labeling of intracellular IFNγ and IL-17A of total colon T cells from WT and CD45E6KO mice after stimulation for 4-5 hours with PMA and Ionomycin and treatment concurrently with Brefeldin A on day 9 post DSS colitis. (D) Graphs of pooled data from three experiments of the frequency and cell number of IFNγ+ and IL-17A+ colon T cells from WT and CD45E6KO mice on day 9 post colitis, n=10-12 mice/genotype. (E) Flow cytometry of GM-CSF production from CD4+ colon T cells on day 9 post DSS from WT and CD45E6KO mice. (F) Graphs of pooled data from two experiments of GM-CSF+ CD4+ T cells in the colon, n=6 mice/genotype. Data represent mean ± SD. Students t test was used for comparison between WT and CD45E6KO mice in D and F; *p<0.05, **p<0.01, ***p<0.001.
were equal to the CD45E6KO mice, and just the numbers of total T cells were less (Figure 3.2D).

Consistent with their enhanced susceptibility to DSS colitis, CD45E6KO mice had increased production of the pro-inflammatory cytokines, IFNγ, IL17A and GM-CSF from colon T cells on day 9 of DSS colitis compared to WT mice (Figure 3.2C-2F). This was also observed in the CD45E9KO mice (data not shown). Furthermore, spleen T cells from DSS-treated CD45E6KO mice have enhanced frequency of IFNγ and IL17A production compared to WT mice (Figure 3.3). This was also observed in the CD45E9KO mice (data not shown). Despite overall reduced numbers of T cells in the spleen of CD45E6KO mice, there were equal numbers of IFNγ+ T cells and increased IL-17A+ T cells in the spleen post DSS (Figure 3.3).

Interestingly, there are increased percentage of Foxp3+ T cells in CD45−/− splenic T cells prior to and post colitis (Figure 3.4A-C). However, this did not translate into increased Foxp3+ Treg cell numbers in the spleen, nor was there any difference in percentage or number of Foxp3+ T cells in the colon prior to and post colitis (Figure 3.4A, 3.4D and 3.4E). Thus the increased severity of colitis observed in the intestine of CD45E6KO mice was not due to a decrease in the Treg population. Overall, these results suggest that CD45−/− T cells are more pro-inflammatory upon inflammation despite their reduced numbers in the spleen and lymph nodes. Interestingly, the increased percentage of Foxp3+ T cells observed in the spleen of CD45E6KO mice may be to try and compensate for the enhanced production of IFNγ and IL-17A in the spleen. CD45−/− colon T cells also have enhanced production of IFNγ and IL-17A but unlike the spleen, the numbers are increased corresponding with enhanced expression of α4β7 and there was no compensatory increase in Tregs.
Figure 3.3. CD45E6KO splenic T cells have increased production of pro-inflammatory cytokines post DSS. (A) Representative flow cytometry labeling of IFNγ and IL17A from T cells in the spleen. Frequency and cell number of IFNγ+ (B) and IL-17A+(C) T cells in the spleen post DSS. Data is pooled from 3 experiments, n=9 mice/genotype. Data is shown as mean± SD. Students t test was used for comparison and *p<0.05.
Figure 3.4. There is no difference in regulatory T cell numbers in CD45KO post DSS. (A) Representative flow cytometry labeling of Foxp3+ regulatory T cells in the spleen and LP of control WT and CD45E6KO and mice on day 9 post DSS. (B) Data from one experiment of frequency and cell number of Foxp3+ Tregs in the spleen in control mice; n=3 mice/genotype. (C) Frequency and cell number of Foxp3+ Tregs in the spleen post DSS; data is pooled from 3 experiments; n=9 mice/genotype. (D) Frequency and cell number of Foxp3+ Tregs in the LP in control mice; n=3 mice/genotype. (E) Frequency and cell number of Foxp3+ Tregs in the LP on day 9 post DSS; data is pooled from 3 experiments; n=9 mice/genotype. Data is shown as mean± SD. Students t test was used for comparison and ***p<0.001.
To assess whether the CD45\(^{-/-}\) innate immune system is also more pro-inflammatory upon DSS treatment, we next evaluated pro-inflammatory cytokine production from colon ILCs. While there was no difference in the frequency and numbers of Lineage Thy1.2+ cells in the colon between CD45E6KO and WT mice, like CD45\(^{-/-}\) T cells, colon CD45\(^{-/-}\) ILCs also had increased percentage and numbers of IFN\(\gamma\) producing cells (Figure 3.5A-C). This was also seen in the CD45E9KO mice (data not shown). Unlike CD45E6KO T cells however, there was no difference in the percentage or numbers of IL17A+ or GM-CSF+ Lineage Thy1.2+ cells in the colon of CD45E6KO compared to WT mice on day 9 of DSS treatment (Figure 3.5A, 3.5D and 3.5E).

Despite the enhanced GM-CSF production by CD45\(^{-/-}\) T cells post colitis, myeloid cell frequency and numbers in the colons of CD45E6KO mice were comparable to WT mice post colitis (Figure 3.5F and 3.5G). However, the percentage and numbers of TNF\(\alpha\) + cells within the CD11c\(^{hi}\)MHCII\(^{hi}\) population were significantly increased in CD45E6KO mice compared to WT mice upon DSS colitis (Figure 3.5F and 3.5H) and this was also observed in the CD45E9KO mice (data not shown).

Together, these data suggest that CD45 negatively regulates the expression of the gut homing molecule, \(\alpha4\beta7\) on T cells and their ability to produce IFN\(\gamma\) and GM-CSF upon DSS induced intestinal inflammation. However, in the presence of an adaptive immune system, the lack of CD45 had a less dramatic impact on innate immune cells although CD45 on ILCs and myeloid cells does seem to negatively regulate production of the pro-inflammatory cytokines, IFN\(\gamma\) and TNF\(\alpha\) respectively.
Figure 3.5. CD45KO mice have enhanced IFNγ production from ILCs and TNFα production from myeloid cells in the colon post colitis. (A) Representative flow cytometry labeling of colon Lineage-Thy1.2+ and their production of IFNγ, IL-17A and GM-CSF from WT and CD45E6KO mice after stimulation for 4-5 hours with PMA and Ionomycin and treatment concurrently with Brefeldin A on day 9 post DSS. Pooled data from at least two experiments of Lin-Thy1.2+ cell frequency and number in the colon (B) and the percentage and number of IFNγ+ (C), IL-17A+ (D) and GM-CSF+ (E) cells within that population, n=6-13 mice/genotype. (F) Representative flow cytometry labeling of colon CD11c+MHCII+ cells and TNFα+ cells within this population from WT and CD45E6KO mice after treatment with Brefeldin A for 4-5 hours on day 9 post DSS. Graphs are pooled data from 3 experiments of the frequency and cell number of CD11c+MHCII+ cells (G) and the percentage and number of TNFα+ cells (H) within this population, n=11-12 mice/genotype. Data represents mean ± SD. Students t test was used for comparison between WT and CD45E6KO mice; *p<0.05.
3.2.2 In the Absence of T cells, CD45 Deficient ILCs have Decreased IL-22 and GM-CSF Production.

It was not clear whether the intestinal adaptive immune system of the CD45\(^{-/-}\) mice was responding to the hyperactive innate immune system or whether the CD45\(^{-/-}\) T cells were intrinsically defective. To answer this question, we generated both CD45RAGE6KO and CD45RAGE9KO mice and induced DSS colitis. Surprisingly, there was no significant difference in weight loss between the RAGKO, CD45E6RAGKO or the CD45E9RAGKO mice upon DSS treatment (Figure 3.6A and 3.6B).

Unlike the CD45E6KO T cells, CD45 was undetectable in the innate immune cells in the CD45E6KO mice. Since there were no differences in weight loss between CD45E6RAGKO and CD45E9RAGKO mice upon inflammation, CD45E9RAGKO mice were used for further analysis, however we did confirm results with CD45E6RAGKO mice as well. Interestingly, the CD45E9RAGKO mice did have shorter colons on day 9 of the experiment but there was no difference in colon cell number post inflammation, suggesting that the inflammation in the intestine of CD45E9RAGKO mice is only subtly enhanced compared to RAGKO mice (Figure 3.6C, 3.6D and 3.7). This was also confirmed in CD45E6RAGKO mice (data not shown). CD45E9RAGKO had comparable colon ILC frequency and number to RAGKO mice but unlike CD45KO mice, CD45E9RAGKO mice did not have increased IFN\(_{\gamma}\) and IL17A production from ILCs upon colitis development (Figure 3.6E, 3.6F and 3.7). Rather, IFN\(_{\gamma}\) and GM-CSF production by Lin\(^{-}\)Thy1.2+NK1.1+ ILCs was reduced in CD45E9RAGKO compared to RAGKO mice on day 9 (Figure 3.6E and 3.6G). This was unexpected because the colons of CD45E9RAGKO upon DSS treatment are significantly shorter than RAGKO mice, suggesting that the inflammation in the colon is more severe in CD45E9RAGKO mice despite no difference
Figure 3.6. CD45RAGKO mice have decreased IFNγ, IL-22 and GM-CSF production from colon ILCs upon colitis. RAGKO, CD45E6RAGKO (A) and CD45E9RAGKO (B) mice were given 3% (w/v) DSS in their drinking water for 0-7 days and then put on water for an additional 2 days. Representative experiment of % loss of body weight is shown and was repeated 5-12 times with at least 3-5 mice per genotype. (C) Colon length of untreated (left) RAGKO and CD45E9RAGKO mice and on day 9 of DSS colitis (right). (D) Pooled data from 5 experiments of colon LP cell number of RAGKO and CD45E9RAGKO mice on day 9 post DSS, n=17 mice/genotype. (E) Representative flow cytometry labeling of IFNγ, IL-17A, IL-22 and GM-CSF from Lineage-Thy1.2+ (left) and Lineage-Thy1.2+NK1.1+ (right) cells in the colon from RAGKO and CD45E9RAGKO mice on day 9 post DSS. Pooled data from two experiments of the frequency and cell number of IFNγ+ and IL-17A+ (F) as well as IL-22+ and GM-CSF+ (G) cells from Lin-Thy1.2+NK1.1+ cells in the colon of RAGKO and CD45E9RAGKO mice on day 9 post DSS, n=5-6 mice/genotype. Data represents mean ± SD. Students t test was used for comparison between WT and CD45E6KO mice; *p<0.05 and ***p<0.001.

Figure 3.7. There is no difference in ILC frequency or number in RAGKO or CD45RAGKO post DSS. (A) Pooled data from at least two experiments of spleen, mLNs and LP cell number of RAGKO and CD45E9RAGKO mice on day 9 post DSS treatment, n=6-31 mice/genotype. (B) Representative flow cytometry labeling of Lineage-Thy1.2+ cells in the colon of RAGKO and CD45E9RAGKO on day 9 post DSS colitis. (C) Pooled data from 4 experiments of frequency and cell number of colon Lin-Thy1.2+ cells and Lin-Thy1.2+NK1.1+ cells from RAGKO and CD45E9RAGKO mice post DSS, n=11-14 mice/genotype. Data is shown as mean± SD. Students t test was used for comparison and **p<0.01.
in systemic inflammation. Since ILC IL-22 has been shown to be protective in DSS colitis (Cox et al., 2012), we assessed whether this cytokine was altered in the CD45E9RAGKO mice upon DSS treatment. Indeed, IL-22 was reduced from LinThy1.2+NK1.1+ ILCs in CD45E9RAGKO mice compared to RAGKO mice on day 9-post colitis induction (Figure 3.6E and 3.6G).

These observations suggest that in the absence of T cells, CD45^/- ILCs have an impaired ability to produce the cytokines IL-22 and GM-CSF and the reduced IL-22 may contribute to enhanced gut inflammation in the CD45RAGKO mice.

3.2.3 In the Absence of T Cells, CD45 Helps Maintain the Myeloid Population in the Gut and Promotes Their Retinoic Acid Production

There have been several studies examining the cross talk between ILCs and myeloid cells in the gut. It has been shown that IL-23 production by myeloid cells promotes IL-22 production by ILCs and in the absence of IL-23, colitis is more severe (Cox et al., 2012). In addition to IL-23 production, DCs have also been shown to be an important source of RA in the gut and it has recently been shown that RA production promotes IL-22 production by ILCs thereby attenuating inflammation in DSS colitis (Mielke et al., 2013). Therefore, we wanted to assess if the reduction in IL-22 production by ILCs in the CD45E9RAGKO mice upon DSS treatment may be due to alterations in myeloid cells. On day 9 of the experiment, CD45E9RAGKO mice consistently had a significantly reduced frequency and less numbers of CD11c^{hi}MHCII^{hi} cells in the colon (Figure 3.8A). This was also observed in CD45E6RAGKO mice (data not shown). Unlike CD45E6KO mice, which had an increased percentage of TNFα producing cells from gut CD11c^{hi}MHCII^{hi} cells upon DSS treatment, there was no difference in TNFα production from gut
Figure 3.8. CD45RAGKO mice have reduced CD11c<sup>hi</sup>MHCII<sup>hi</sup> cells in the colon and reduced production of RA from DCs in the mLNs post DSS. (A) Flow cytometry (left) and frequency and cell number (right) of CD11c<sup>hi</sup>MHCII<sup>hi</sup> cells and TNFα<sup>+</sup> cells within this population in the colon of RAGKO and CD45E9RAGKO mice on day 9 post DSS. Data is pooled from at least 2 experiments, n=8-12 mice/genotype. (B) Flow cytometry (left) and frequency and cell number (right) of CD11c<sup>hi</sup>MHCII<sup>hi</sup> cells in the mLNs and their expression of ALDEFLUOR (left) as well as the negative control (DEAB) for ALDEFLUOR post DSS. (E) Frequency and cell number of DCs and ALDEFLUOR+ DCs in the mLNs post DSS. Data is pooled from at least 2 experiments and represents mean ± SD, n=5-10 mice/genotype. Students t test was used for comparison and *p<0.05.
CD11c<sup>hi</sup>MHCII<sup>hi</sup> cells between RAGKO and CD45E9RAGKO mice post DSS (Figure 3.8A). Therefore, we evaluated RA production by DCs post DSS treatment using ALDEFLUOR, a synthetic substrate that accumulates in cells after cleavage and is proportional to active aldehyde dehydrogenases, enzymes involved in RA production. There was minimal expression of ALDEFLUOR from colon CD103+ myeloid cells on day 9 of DSS colitis in the RAGKO and CD45E9RAGKO mice (Figure 3.9). Nevertheless, frequency and cell numbers of ALDEFLUOR+CD103+ myeloid cells was reduced in CD45E9RAGKO mice (Figure 3.9). Notably, there was a significant reduction in expression of ALDEFLUOR from DCs in the mLNs of CD45E9RAGKO post DSS treatment, the majority of which express CD103 (Figure 3.8B).

There was no difference in DC frequency in the mLNs between the CD45E9RAGKO and RAGKO mice on day 9, suggesting that there is no migratory defect in DCs from the colon to the lymph nodes and that the reduction in CD11c<sup>hi</sup>MHCII<sup>hi</sup> cells in the colon may be due to local cues from the gut of the CD45E9RAGKO mice post DSS (Figure 3.8B).

Overall, these results suggest that CD45 is required to maintain the DC population in the colon during colitis and that CD45 on DCs in the mLNs regulates RA production upon inflammation.

### 3.2.4 The Absence of CD45 on the Innate Immune System Delays CD45 Sufficient Effector T Cell Homing and Attenuates Colitis

Based on the previous results, the exacerbated inflammation observed in the CD45KO mice upon DSS colitis is primarily due to CD45<sup>−/−</sup> T cell-intrinsic effects leading to enhanced pro-inflammatory cytokine production and enhanced gut homing (Figures 3.1 and Figure 3.2).
Figure 3.9. Colon CD103+ myeloid cells in CD45RAGKO mice have reduced production of RA. (A) Flow cytometry labeling of ALDEFLUOR+ cells within CD11c\textsuperscript{hi}MHCII\textsuperscript{hi}CD103+ cells in the colon as well as the negative control (DEAB) for ALDEFLUOR post DSS. (B) Frequency and cell number of CD11c\textsuperscript{hi}MHCII\textsuperscript{hi}CD103+ ALDEFLUOR+ cells in the colon post DSS. Data is pooled from two experiments, n=6 mice/genotype. Data is shown as mean± SD. Students t test was used for comparison and *p<0.05.
CD45+ adaptive immune cells also caused the CD45− innate immune system to be hyperactive since CD45E9RAGKO mice did not have enhanced TNFα production from myeloid cells nor increased IFNγ from ILCs compared to the RAG mice, unlike CD45KO mice compared to WT mice. Indeed, levels were equal or lower for the proinflammatory cytokines made by ILCs in the CD45E9RAGKO mice compared to the RAG mice. These results suggest that adaptive immune cells can cross talk with the innate immune cells to promote cytokine production by ILCs and RA expression by myeloid cells. To assess whether CD45 sufficient T cells could restore ILC cytokine production and/or RA production by DCs in CD45RAGKO mice, we adoptively transferred polyclonal T cells from naïve WT mice into RAGKO and CD45E9RAGKO mice and then induced DSS colitis. Surprisingly, there was a significant delay in weight loss from days 5-8 in the CD45E9RAGKO mice upon transfer of CD45+ T cells and DSS treatment (Figure 3.10A). This was also observed in CD45E6RAGKO mice (data not shown). This was opposite to the enhanced weight loss and more severe colitis observed in the CD45KO mice compared to the WT mice. Consistent with the delay in systemic inflammation, on day 8 post DSS treatment, CD45E9RAGKO mice had longer colons and reduced cell number suggesting lower intestinal inflammation in these mice compared to RAGKO mice (Figures 3.10B and 3.10C).

The transfer of CD45+/+ polyclonal T cells did not restore the reduction in frequency or number of CD11cahiMHCIIahi cells in the colon of CD45E9RAGKO post DSS treatment (Figure 3.10D). Since there was no change in myeloid cell frequency or number in the colon of CD45E9RAGKO mice upon DSS colitis, we next examined the colon for the polyclonal T cells that were adoptively transferred. On day 8-post colitis induction, CD45E9RAGKO mice had significantly reduced frequency and numbers of both CD4+ and CD8+ T cells in the colon (Figure 3.10E and 3.10F). In addition to reduced T cells in the colon, CD45E9RAGKO mice also
The transfer of CD45 sufficient WT polyclonal T cells reduces the severity of DSS colitis in CD45RAGKO mice. (A) Mice were injected with 3-5 x 10^6 T cells I.V. on day 0 and were given 3% (w/v) DSS in their drinking water for 0-7 days and then put on water for an additional 1-2 days. Graph is representative of % loss of body weight in RAGKO and CD45RAGKO mice and conducted 4 times with at least 2-3 mice/genotype. Pooled data from at least 3 experiments of colon length (B) and colon cell number (C) of RAGKO and CD45RAGKO mice on day 8 post DSS and T cell injection, n=9-14 mice/genotype. (D) Pooled data from 3 experiments of colon CD11c<sup>hi</sup>MHCII<sup>hi</sup> cell frequency and number on day 8 post DSS and T cell injection in RAGKO and CD45RAGKO mice, n=9-10 mice/genotype. Flow cytometry (E) and pooled data from at least 3 experiments of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from the colon of RAGKO and CD45E9RAGKO mice on day 8 post DSS and T cell injection, n=9-10 mice/genotype. Flow cytometry (G) and pooled data from at least 4 experiments (H) of CD4<sup>+</sup> and CD8<sup>+</sup> T cells from the mLNs of RAGKO and CD45E9RAGKO mice on day 8 post DSS and T cell injection, n=13-16 mice/genotype. Data represents mean ± SD. Students t test was used for comparison between RAGKO and CD45E9RAGKO mice; *p<0.05, **p<0.01, ***p<0.001.
had reduced T cells in the mLNs (Figure 3.10G and 3.10H). These results suggest that the reduction of T cells in the gut of CD45E9RAGKO mice upon DSS colitis ameliorates disease in these mice compared to RAGKO mice. A recent study demonstrated that upon the transfer of polyclonal CD45\(^{+/+}\) T cells into naïve CD45E9RAGKO and RAGKO mice, there was reduced lymphopenia-induced proliferation of the transferred T cells in CD45E9RAGKO mice resulting in lower frequency and number of T cells in the spleen and peripheral lymph nodes on day 7 post transfer (Saunders et al., 2014). However upon inflammation induced by DSS, there was no difference in T cell frequency or number in the spleen between CD45E9RAGKO and RAGKO mice (Figure 3.11) suggesting that lymphopenia-induced proliferation is not a contributing factor to the reduction of colon T cells observed in CD45RAGKO mice. Another possibility that could contribute to the delay in disease observed in the CD45RAGKO mice could be the enhanced induction of Foxp3+ T cells. However, there was no significant differences in the percentage of Foxp3 within CD4+ or CD8+ T cells in the spleen, mLN and LP, and there were actually less Tregs in the colon of the CD45E9RAGKO mice (Figure 3.12). This indicates that increased numbers of regulatory T cells are not the reason for the reduced colitis observed in the CD45E9RAGKO mice.

Given that DCs from the mLN of CD45E9RAGKO mice upon DSS treatment had reduced capacity for RA expression, we wanted to evaluate whether upon inflammation insufficient RA could be reducing effector T cell homing to the intestine. While there have been several studies on the importance of RA as an anti-inflammatory factor in the intestine promoting regulatory T cell development at the expense of Th17 cells, there is also evidence for RA in inducing gut homing of effector Th1 cells (Brown et al., 2015; Hall et al., 2011). In the mLN post DSS treatment, the transferred CD45\(^{+/+}\) CD4+ and CD8+ T cells in CD45E9RAGKO mice
Figure 3.11. There is no difference in homeostatic proliferation in the spleen between RAGKO and CD45RAGKO mice post DSS. (A) Representative flow cytometry labeling of CD4+ and CD8+ T cells in the spleen of RAGKO and CD45E9RAGKO mice post DSS. Cell number and frequency of CD4+ (B) and CD8+ (C) T cells in the spleen post DSS. Data is pooled from at least 3 experiments, n=10 mice/genotype. Data is shown as mean± SD. Students t test was used for comparison.
Figure 3.12. Upon the transfer of CD45 sufficient WT polyclonal T cells and DSS colitis induction, the delay in colitis progression is not due to enhanced Foxp3+ Tregs in CD45RAGKO mice. Cell number and frequency of CD4+ (A) and CD8+ (B) Foxp3+ Tregs in the spleen of RAGKO and CD45E9RAGKO mice on day 9 post DSS. Cell number and frequency of CD4+ (C) and CD8+ (D) Foxp3+ Tregs in the mLNs post DSS. Cell number and frequency of CD4+ (E) and CD8+ (F) Foxp3+ Tregs in the LP post DSS. Data is pooled from 2 experiments, n=6 mice/genotype. Data is shown as mean± SD. Students t test was used for comparison and *p<0.05 and ***p<0.001.
had significantly reduced frequency and expression of α4β7 (Figure 3.13A-E) and CD8+ T cells also have reduced expression of the gut homing chemokine receptor, CCR9 (Figure 3.13A and 3.13E).

Altogether, these results suggest that the reduced capacity of CD45⁻ DCs to produce RA during inflammation has significant consequences on CD45⁺ T cell effector homing and ultimately leads to reduced T cell driven inflammation.

### 3.2.5 Exogenous GM-CSF and Retinoic Acid Restores Intestinal Effector CD45⁺ T Cells and Promotes Inflammation in CD45RAGKO Mice

To test whether it was the reduced RA that was leading to the decreased T cell homing and gut inflammation in CD45RAGKO mice, we injected all-trans retinoic acid. Upon the injection of polyclonal CD45⁺ T cells and induction of DSS colitis, we injected all-trans RA every other day from day 1 of the experiment, as had been done previously (Mielke et al., 2013). Providing exogenous RA to CD45E9RAGKO mice caused similar weight loss and similar colon lengths between these mice and RAGKO mice, while CD45E9RAGKO mice injected with vehicle only still had delayed weight loss and longer colons compared to RAGKO mice (Figure 3.13F). This result confirms that in an inflammatory setting, CD45E9RAGKO mice have insufficient RA production and that by providing it exogenously, colitis is worse, in line with an inflammatory role for RA for inducing homing molecules on effector T cells and promoting their migration to the gut (Hall et al., 2011).

GM-CSF production by ILCs and intestinal stromal cells has been shown to induce the production of RA by intestinal DCs (Mortha et al., 2014; Vicente-Suarez et al., 2015). Given that we have shown that CD45⁻ ILCs in the colon make less GM-CSF upon inflammation, we next
Figure 3.13. Exogenous RA and GM-CSF enhances DSS colitis in CD45RAGKO mice. (A-E) Representative flow cytometry labeling and pooled data from 4 experiments of α4β7 and CCR9 expression on CD4+ and CD8+ T cells in the mLNs of RAGKO and CD45E9RAGKO mice on day 8 post DSS and T cell injection, n=11-13 mice/genotype. Mice were injected with 3-5 x10^6 T cells I.V. on day 0 and were given 3% DSS (w/v) in their drinking water for 0-7 days and then put on regular water for an additional 1-2 days and 200 µg/mouse of RA or vehicle (F) or 5 µg/mouse of GM-CSF or PBS (G) was injected I.P. into mice every other day from day 1. Representative experiment from two conducted of % loss of body weight is shown, n=3-5 mice/genotype. Pooled data from two experiments of colon length post DSS with RA or vehicle (F) or GM-CSF or PBS (G), n=5-6 mice/genotype. (H) Pooled data from two experiments of frequency and cell numbers of CD4+ and CD8+ T cells in the colon post DSS + polyclonal T cell transfer and GMCSF or PBS injection. (I) Pooled data from two experiments of cell frequency and number of ALDEFLUOR expression from CD11c^hiMHCII^hiCD103^+ cells from the mLNs post DSS + polyclonal T cell transfer and with or without GM-CSF injection. Data is represented as mean± SD. ANOVA was used for comparison from F-I and *p<0.05, **p<0.01, ***p<0.001.
assessed whether GM-CSF could also restore inflammation in CD45E9RAGKO mice if provided exogenously. Again, upon the injection of polyclonal CD45^{+/+} T cells and induction of DSS colitis, we injected recombinant mouse GM-CSF every other from day 1 of the experiment. Similarly to the RA injections, providing exogenous GM-CSF also restored the weight loss and colon lengths of CD45E9RAGKO mice to RAGKO mice upon DSS treatment and T cell injection (Figure 13.3G). The injection of GM-CSF into CD45RAGKO mice also restored the frequency and number of T cells in the colon (Figure 13.3H). Finally, exogenous GM-CSF re-established the expression of ALDEFLUOR in CD103^+ DCs in the mLNs of CD45E9RAGKO mice upon inflammation (Figure 13.3I).

Thus, providing exogenous RA and GM-CSF demonstrates the importance of these factors in inducing appropriate effector T cell homing to the intestine upon inflammation and demonstrates a positive role for CD45 in the production of RA and GM-CSF by innate immune cells.

### 3.3 Discussion

In this report we show a novel role for CD45 in regulating immune cell functions in intestinal inflammation. CD45 is well-known for regulating Src family kinase activity in T and B cells and other studies have shown a regulatory role for CD45 in cytokine signaling in mast cells and TLR induced cytokine production by DCs in vitro (Cross et al., 2008; Irie-Sasaki et al., 2001; Piercy et al., 2006). Here we show that CD45 regulates cytokine production and the induction of T cells homing molecules in a mouse model of intestinal colitis. We demonstrate that CD45 has a largely negative effect on T cells by limiting pro-inflammatory cytokine production and gut T cell homing. Conversely, CD45 has a largely positive effect on innate
immune cells in response to inflammatory stimuli in the gut by promoting ILC GM-CSF and IL-22 production and by maintaining myeloid cells in the gut. In DSS colitis, the loss of CD45 results in the upregulation of the gut homing molecule α4β7 on effector T cells, such that effector CD45^−/− T cells exhibit enhanced expression and accumulate in the gut upon DSS induced colitis. CD45 also down regulates pro-inflammatory cytokine production by effector T cells in the colon. In the absence of an adaptive immune system, CD45 on ILCs promotes their production of IFNγ, GM-CSF and IL-22 upon inflammation. Reduced GM-CSF production by the CD45^{−/−} ILCs contributes to the decreased production of RA by dendritic cells as exogenous GM-CSF rescued RA levels, and reduced GM-CSF may also lead to reduced myeloid cells in the inflamed colon of CD45^{−/−} mice. The loss of inflammatory cytokines appears to be balanced by the loss of IL-22, which has been shown to have a protective role in the gut (Bishop et al., 2014; Cox et al., 2012; Zenewicz et al., 2008), such that there is no large difference in intestinal inflammation between the CD45RAGKO and RAGKO mice, until CD45^{+/+} T cells are added, then the reduced levels of RA results in reduced trafficking of effector T cells to the intestine which results in reduced inflammation.

To support these observations, a recent report showed that GM-CSF production by ILCs is important for the maintenance of macrophages and DCs in the gut (Mortha et al., 2014). GM-CSF^{−/−} mice had reduced myeloid cells in the intestine that made inadequate RA and these mice were unable to induce oral tolerance as a result of insufficient conversion of naïve T cells to regulatory T cells (Mortha et al., 2014). In DSS-induced colitis, CD45RAGKO mice have a similar phenotype to GM-CSF^{−/−} mice; CD45RAGKO mice have reduced myeloid cells in the inflamed gut and lower RA production by the myeloid cells locally in the intestine and mLNrs. There was also significantly reduced numbers of both effector CD4 and CD8 T cells in the
intestine of CD45RAGKO mice which led to reduced DSS induced inflammation in these mice compared to the RAGKO mice. This could not be explained by a change in Tregs, which were also lower in CD45RAGKO mice. It is clear that RA has multiple roles in the gut. It has been shown by various groups that RA can induce Treg conversion but its role in inflammation is more ambiguous. One report shows that RA production promotes IL-22 production by ILCs and γδ T cells and ultimately protects mice from DSS colitis (Mielke et al., 2013) and our data supports this. Conversely, another report shows that RA production is necessary for expression of gut homing receptors on effector CD4+ T cells and when WT mice are given a vitamin A deficient diet and infected with a parasite, *Toxoplasma gondii*, or when mice deficient for RA receptor α are infected with this parasite, there is insufficient effector CD4+ T cells in the intestine and inefficient clearance of the parasite (Hall et al., 2011). In addition, RA also induces gut tropic molecules on Tregs (Coombes et al., 2007). We demonstrate that RA can promote inflammation in DSS-induced colitis when T cells are present and that in addition to being beneficial in bacterial clearance, RA can also be detrimental to the host depending on the type of inflammation. We also show that CD45 expression can promote RA production by CD103+ DCs. GM-CSF production by ILCs and also by stromal cells has been shown to be required in the gut during homeostasis to maintain the macrophage and DC populations in the gut, but also to promote RA production by DCs (Mortha et al., 2014; Vicente-Suarez et al., 2015). Since we do not see a reduced myeloid population in the colon prior to colitis, we think that CD45 is important for inflammation induced GM-CSF levels in the gut. We show that upon DSS induced colitis exogenous GM-CSF restores RA production by CD45+ CD103+ DCs, but unlike in homeostasis, where Treg conversion predominates, in inflammation this results in increased effector T cells in the gut.
Our results also show a new role for CD45 on the innate immune system upon inflammation in promoting GM-CSF production by ILCs which can stimulate DCs to produce RA and promote effector T cell homing. What is less clear is whether CD45 on myeloid cells influences GM-CSF production by ILCs, perhaps by regulating microbial induced IL-1β production by gut macrophages or whether CD45 deficiency on ILCs themselves regulates GM-CSF production by ILCs. It has been shown that the tyrosine kinase, Lyn, is important for regulating IL-22 production by ILCs in DSS colitis and since CD45 is a regulator of Lyn (Johnson et al., 2012), it is possible that CD45 may regulate Lyn and through Lyn control IL-22 and GM-CSF production by ILCs (Bishop et al., 2014).

What is note-worthy is that when DSS colitis is induced in the CD45KO mice where endogenous T cells are present, there is no deficiency in effector T cell homing or macrophage and DC frequency and number in the gut as compared to when CD45+/+ T cells are transferred into CD45RAGKO mice. The CD45−/− T cells have increased expression of the gut homing molecule, α4β7, which may help compensate for any reduced homing signals produced by the innate immune cells. As well, in the inflammed intestine, CD45−/− T cells themselves make more GM-CSF than WT T cells and this may be sufficient to overcome other cell-types such as ILCs or stromal cells that are not producing sufficient GM-CSF to promote myeloid cell RA production upon inflammation. A caveat in these above models is the lack of histology to score intestinal inflammation and therefore, future studies should assess disease to confirm the colon length results.

Extensive research into the role of cytokines in the gut has led to some effective treatments for IBD in humans. Anti-TNFα therapy in particular has been successful in treating
IBD and an antibody for the common subunit for IL-23 and IL-12 has also shown to be effective in clinical trials (Sandborn et al., 2012; van Dullemen et al., 1995). However, there have been disappointments with anti-cytokine therapy as well; antagonizing IFNγ or IL-17A were not effective in improving IBD and providing recombinant IL-10 was also shown to be ineffective (Hueber et al., 2012; Reinisch et al., 2006; Tilg et al., 2002). Exploring other avenues of treatment for Crohn’s disease or ulcerative colitis will be key for patients who do not respond to these anti-cytokine treatments. Our study suggests that targeting CD45 may provide another avenue of treatment for IBD since we have shown it to be a key regulator of homing of gut effector T cells and for regulating GM-CSF and RA production in the intestine. We corroborate the evidence that GM-CSF promotes RA production and we show the consequence of this pathway in inflammation. We go on further to provide evidence that CD45 can regulate this mechanism upon inflammation and because of the multiple roles CD45 plays in the gut that it could be a target for novel immunotherapies for IBD.
Chapter 4: CD45 on Intestinal Myeloid Cells Limits Gut Inflammation but Promotes Systemic Disease and Erythrocyte Maturation

4.1 Introduction and Rationale

The T cell transfer model of colitis has been key in identifying the various roles of T cells and their cytokines in the pathogenesis of IBD (Groux et al., 1997; O'Connor et al., 2009; Powrie et al., 1994). In addition to the role of T cells, intestinal myeloid cells and their cytokines have also been implicated for their roles if perpetuating and regulating inflammation (Ahern et al., 2008; Coombes and Powrie, 2008). The T cell transfer model of colitis involves the separation of naive T cells from regulatory T cells and their injection into immunodeficient mice such as RAG mice (Powrie et al., 1993).

Since the transfer of polyclonal T cells into RAGKO and CD45RAGKO mice resulted in less severe DSS-induced colitis in CD45RAGKO mice, I wanted to assess if this would also be observed with the T cell transfer model. In addition, I wanted to assess the role of CD45 in non-lymphoid cells in vivo to see if the absence of CD45 results in enhanced cytokine production in vivo or if in the absence of an adaptive immune system the loss of CD45 on innate immune cells would result in minimal pro-inflammatory cytokine secretion by myeloid cells.

Interestingly, the development of IBD in an individual is not only restricted to inflammation in the intestine but also results in other various extraintestinal complications such as weight loss and anemia (Danese and Fiocchi, 2011). As many as one-third of IBD patients develop anemia, which may result from either iron deficiency due to malabsorption, or from ineffective erythropoiesis and shortened red blood cell (RBC) survival, also known as anemia of
chronic diseases (ACD) (Gasche et al., 2004; Wilson et al., 2004). The development of intestinal inflammation is also often associated with weight loss in humans and almost always in animal models. As inflammation gets more severe, so does weight loss and depending on the particular model, weight loss to a pre-determined endpoint can occur as soon as 7 days in the DSS model of colitis or may take up to several months in the T cell transfer model of colitis. The rate of weight loss in the T cell transfer model varies greatly depending on the particular animal unit likely due to alterations in the microbiota of mice but can also be due to differences in diet, bedding and other environmental differences. The occurrence of *Helicobacter* species specifically is common in commercial and academic institutions throughout North America and Europe (Chichlowski and Hale, 2009; Shames et al., 1995) and *Helicobacter hepaticus* has been shown to coincide with colon inflammation in immunodeficient or susceptible strains of mice (Kullberg et al., 2002). In addition, T cell clones specific for *H. hepaticus* from infected mice transferred into *Helicobacter hepaticus* positive RAG deficient mice cause colitis whereas a Th1 clone specific for a control species, *Schistosoma mansoni*, transferred into these same *H. hepaticus* positive RAG deficient mice did not cause disease (Kullberg et al., 2003).

Furthermore, the microbial status of mice in particular animal units can accelerate disease. For instance, RAG deficient mice that are known to be negative for *H. hepaticus* lose 10-20% of their original weight and develop intestinal inflammation by week 8 (Griseri et al., 2010; Izcue et al., 2008), whereas RAG deficient mice that are *Helicobacter hepaticus* positive lose the same amount of weight and develop intestinal inflammation in only 2-3 weeks (Kamanaka et al., 2011). Therefore, not only is the presence of a particular microbe in mice necessary to cause disease but it also affects the rate of disease development upon the T cell transfer induction of colitis.
In this project, I wanted to assess first if the transfer of naïve CD4+ T cells would also result in less severe disease in CD45RAGKO mice as the transfer of polyclonal T cells does in the DSS-induced model of colitis. Furthermore, CD45RAGKO mice have been bred and sustained in several difference animal units that are known to be positive and negative for certain microbiota such as *Helicobacter hepaticus*. Therefore, I wanted to assess if the change in the microbiota of these animals would alter the progression or development of T cell transfer colitis. Based on the results with DSS-induced colitis, I hypothesize that in T cell transfer colitis, CD45RAGKO will also develop less severe disease.

4.2 Results

4.2.1 Loss of CD45 on Innate Immune Cells Delays Weight Loss Upon T Cell Transfer Colitis

It has previously been shown that CD45 regulates pro-inflammatory cytokine production by in-vitro derived DCs in a TLR dependent manner (Cross et al., 2008; Piercy et al., 2006). We therefore wanted to assess whether in vivo, enhanced cytokine production by CD45−/− myeloid cells results in increased inflammation. Using the T cell transfer model, naïve CD4+CD25− CD45RBhigh T cells were isolated and these T cells were injected into RAGKO and CD45RAGKO mice in three different animal units. Mice in the different animal units were genetically identical but had different microbiota depending on the animal unit. The mice in the Wesbrook Animal Unit (WAU) were specific pathogen free (SPF) but *Helicobacter* species positive. Once these mice were transferred into the pre-Containment Level 1 (pCL1) of the Centre for Disease Modeling, mice were SPF but tested positive for *Spironucleus muris*. In addition, RAGKO and CD45RAGKO mice were re-derived into the Centre for Disease
Modeling (CDM) and tested negative for all *Helicobacter* species, *Spironucleus* and all other tested pathogens. Despite the differences in the microbiota in each animal unit, upon the induction of T cell colitis, CD45RAGKO mice had a significant delay in weight loss in each unit (Figure 4.1A-C). Interestingly, the differences in each animal unit affected the onset of the disease. RAGKO mice in pCL1 started to lose weight from days 4-6 and succumbed to disease by day 16 (Figure 4.1A). Mice from the WAU started to lose weight from day 14-21 and succumbed to disease near day 49 (Figure 4.1B). Re-derived RAGKO mice from CDM, started to lose weight 25-30 days after mice from the WAU or pCL1 did and finally succumbed to disease near day 77 (Figure 4.1C). In each case, CD45RAGKO mice showed a delayed onset of T cell transfer colitis. These results indicate that CD45 deficiency on innate immune cells delays the onset of weight loss during T cell colitis, and the delay in weight loss in CD45 deficient mice is independent of the microbiota present.

4.2.2 CD45 on Innate Immune Cells Delays Systemic Inflammation but Does Not Induce Increased Regulatory T Cells

After T cell colitis induction, mice were compared at the time point when RAGKO mice reached their weight loss endpoint (RAGKO endpoint). In addition, CD45RAGKO mice were analyzed when they reached their own weight loss endpoint (CD45RAGKO endpoint). Prior to and post colitis induction, CD45RAGKO mice have increased cell number in the spleen (Figure 4.2A). Upon the induction of T cell colitis, in the spleen, CD45RAGKO mice at the RAGKO endpoint have decreased frequency of T cells in the spleen, however T cell numbers are comparable (Figure 4.2B). When CD45RAGKO mice reach their weight loss endpoint, T cell frequency in the spleen was comparable to RAGKO mice (Figure 4.2B). Unlike the spleen, at
Figure 4.1. **CD45RAGKO mice have delayed weight loss upon T cell transfer colitis.** 4x10^5 CD4+CD25-CD45RB^high^ cells were isolated from the spleen of C57Bl/6 mice and injected i.p. into RAGKO and CD45RAGKO in the (A) pre-Containment Level 1 of the Centre for Disease Modeling (B) Wesbrook Animal Unit and (C) Containment Level 1 of the Centre for Disease Modeling. Mice were weighed every 3-7 days and are representative data from at least 2 experiments. Data represent mean ± S.D. * p<0.05, **p<0.01, ***p<0.001.
Figure 4.2. CD45RAGKO mice have reduced systemic T cells and production of myeloid cell TNFα. (A) Pooled data of at least 4 experiments of the total cell number of splenocytes in RAGKO (white) and CD45RAGKO (black) mice prior to and post colitis induction at the RAGKO endpoint and at the CD45RAGKO endpoint (striped), n=12-38. (B) Pooled data of at least 4 experiments of TCRβ+ cell frequency and numbers in the spleen analyzed by flow cytometry at the RAGKO endpoint and at the CD45RAGKO endpoint, n=12-36. (C) Pooled data of at least 3 experiments of the total cell number of cells in the mLNs in RAGKO and CD45RAGKO mice prior to and post colitis induction at the RAGKO endpoint and at the CD45RAGKO endpoint n=10-27. (D) Pooled data of at least 3 experiments of TCRβ+ cell frequency and numbers in the mLNs analyzed by flow cytometry at the RAGKO endpoint.
endpoint and at the CD45RAGKO endpoint, n=10-25. (E) Representative flow cytometry staining and ICS and graphs of TCRB+CD4+ cells in the spleen and their Foxp3+, IFNγ+ and IL-17A+ expression in the RAGKO and CD45RAGKO mice at the RAGKO endpoint post colitis. Graphs are of pooled data of the frequency and cell numbers from at least 3 experiments, n=10-11. (F) Pooled data of 2 experiments of serum TNFα production from RAGKO and CD45RAGKO mice at the RAGKO endpoint, n=6. (G) Representative ICS flow cytometry and graphs of CD11b+TNFα+ cells in the spleen of RAGKO and CD45RAGKO mice at the RAGKO endpoint post colitis. Graphs are of pooled data of the frequency and cell numbers from 2 experiments, n=8. Data represent mean ± S.D. * p<0.05, **p<0.01, ***p<0.001.
either the RAGKO endpoint or the CD45RAGKO endpoint, there was no difference in frequency of T cells in the mLNs but the numbers of cells in the mLNs at the RAGKO endpoint in CD45RAGKO mice were markedly reduced and eventually recovered upon their own weight loss endpoint (Figure 4.2C,D). Consistent with the reduction of T cells in the spleen and mLNs in CD45RAGKO mice at the RAGKO endpoint, T cell frequency in the blood of CD45RAGKO was also reduced at early time points but increased and approached RAGKO mice levels as colitis progressed (Figure 4.3).

Regulatory T cells are known to be potent suppressors of effector T cell proliferation and pro-inflammatory cytokine production (Sakaguchi et al., 2008). To assess whether effector T cell function was being limited by regulatory T cells, the cytokine production and Foxp3 expression of T cells in the spleen were evaluated at the RAGKO endpoint. At this time point, while there was reduced frequency of T cells in the spleen, there was no difference in Foxp3 expression between RAGKO and CD45RAGKO mice (Figure 4.2E). Notably, T cells in the spleen of CD45RAGKO mice actually have enhanced IL-17A production and equal IFNγ production compared to RAGKO mice (Figure 4.2E). There was also no difference in IFNγ+ and IL-17A+ T cell numbers in the spleen at the RAGKO endpoint suggesting that the delay in systemic disease observed in CD45RAGKO mice upon T cell transfer colitis was not due to decreased numbers of effector T cells in the spleen. Since there were no phenotypic or functional alterations in the transferred effector T cells to correspond with the delay in weight loss observed in CD45RAGKO upon colitis, we next assessed whether innate cell cytokines were altered upon colitis development.
Figure 4.3. 45RAGKO mice have delayed TCRB+CD4+ frequency in the peripheral blood upon T cell transfer colitis. Representative flow cytometry stains of TCRB+CD4+ cells in the peripheral blood of RAGKO and CD45RAGKO mice days 10, 13, 16 and 20 after T cell colitis induction (left). Pooled data of 2 experiments of T cell frequency in the peripheral blood, n=7. Data represent mean ± S.D.
In the spleen, while there was no significant difference in pro-inflammatory cytokine production from spleen explants between RAGKO and CD45RAGKO mice at the RAGKO endpoint, there was a trend for decreased TNFα and IL-6 production (Figure 4.4). To further characterize the reduction of TNFα and to determine whether innate cells produce it, intracellular stain (ICS) for TNFα showed that CD45−/− CD11b+ cells have decreased expression for TNFα at the RAGKO endpoint (Figure 4.2G). In support of reduced systemic inflammation at the RAGKO endpoint, CD45RAGKO mice also had significantly reduced levels of TNFα in the serum (Figure 4.2F). These data demonstrate that CD45 on innate immune cells limits systemic inflammation induced by the transfer of CD45 sufficient naïve T cells. The delay of systemic inflammation was however not dependent on enhanced Tregs but correlated with reduced T cell frequency in the spleen and a reduction in pro-inflammatory cytokines by CD45−/− innate immune cells.

4.2.3 CD45RAGKO Mice have Comparable Intestinal Inflammation to RAGKO mice

Upon T Cell Transfer Colitis

Weight loss in mouse models of colitis is commonly used as an indicator of intestinal inflammation, so we fully expected to observe a decrease in gut inflammation of CD45−/− mice. To our surprise, when the intestinal inflammation was evaluated in the CD45RAGKO mice at the RAGKO endpoint, the severity of inflammation was comparable to RAGKO mice as assessed by colon weight/length, histology and leukocyte infiltration (Figure 4.5A-D).

Interestingly, when CD45RAGKO mice do reach their weight loss endpoint, their colon weight per length was significantly worse than the RAGKO and CD45RAGKO mice at the RAGKO endpoint (Figure 4.5B). Histology on the rectal end of the large intestine at the RAGKO endpoint
Figure 4.4. CD45RAGKO mice have reduced trend for IL-6 and TNFα from spleen explants post colitis induction at the RAGKO endpoint. Weighed spleens were cultured overnight in complete media at 37°C and supernatants from culture were analyzed with a flowcytomix kit to assess production of (A) IL-6, (B) GM-CSF and (C) TNFα. Graphs represent pooled data from 2 experiments, n=5-6. Data represent mean ± S.D.
Figure 4.5. CD45RAGKO mice have equivalent intestinal inflammation at the RAGKO endpoint. (A) Representative pictures of the large intestine of control and colitis mice at the RAGKO endpoint. (B) Pooled data of at least two experiments of the colon weight/length of RAGKO and CD45RAGKO mice at the RAGKO endpoint.
and of the 45RAGKO mice at their weight loss endpoint, n=5-12. (C) Representative rectal colon cross-sections (left) of control and colitis RAGKO and CD45RAGKO mice at the RAGKO endpoint stained with hematoxylin and eosin and pooled data (right) from at least 3 experiments of the histology scores prior to and post colitis at the RAGKO endpoint of RAGKO and CD45RAGKO mice, n=10-17. (D) Pooled data of at least 3 experiments of the total cell number in the colon prior to and post colitis at the RAGKO endpoint and at the CD45RAGKO endpoint, n=10-27. (E) Representative flow cytometry staining (left) of TCRB+CD4+ cells in the colon of mice post colitis induction at the RAGKO endpoint and pooled data of at least 2 experiments of colon T cell frequency and cell number post colitis at the RAGKO endpoint, n=17-33. (F) Representative ICS (left) of colon T cell IFNγ, IL-17A and Foxp3 expression post T cell transfer colitis and pooled graphs (right) of cell frequency and number of T cell IFNγ+, IL-17A+, Foxp3+ cells in the colon post colitis. Data are pooled from at least 2 experiments, n=5-18. Data represent mean ± S.D. * p<0.05, **p<0.01, ***p<0.001.
also confirmed that CD45RAGKO mice have equal intestinal inflammation despite minimal weight loss at this stage. (Figure 4.5C). Furthermore, the total cell number in the colon at the RAGKO endpoint was comparable between RAGKO and CD45RAGKO mice and significantly enhanced in the CD45RAGKO mice when they reached their weight loss endpoint, suggesting that leukocyte infiltration into the colon was equivalent at the RAGKO endpoint and enhanced once the CD45RAGKO reach their weight loss endpoint (Figure 4.5D). Strikingly, these results demonstrate that even though CD45RAGKO mice have delayed progression and development of systemic inflammation, the development of intestinal inflammation upon T cell transfer colitis was equal to that of the RAGKO mice and this was dissociated from the systemic disease. In addition, as the CD45RAGKO mice lose weight, intestinal inflammation progressed and became more severe than the RAGKO mice at equivalent weight loss endpoints. This demonstrates that CD45 plays an important role in systemic spread of inflammation.

4.2.4 There is No Difference in Intestinal T Cells in CD45RAGKO Mice Compared to RAGKO Mice Upon T Cell Transfer Colitis

To address the contrast between the systemic and local intestinal inflammation in the CD45RAGKO mice upon T cell transfer colitis, we assessed the number and frequency of T cells in the colon LP. We hypothesized that T cells in the CD45RAGKO mice had either been induced to become more pro-inflammatory and restricted to the intestine due to local intestinal cues or accumulated in the CD45RAGKO colon upon colitis induction and were unable to spread systemically thereby delaying inflammation and weight loss. We found no evidence for effector T cell accumulation in the colon (Figure 4.5E). At the RAGKO endpoint, there was a slight but significant reduction in frequency of colon T cells in the CD45RAGKO mice and no difference
in number (Figure 4.5E). Next, to evaluate whether the T cells in the CD45RAGKO mice were more pro-inflammatory in the gut, we assessed IFNγ and IL-17A by ICS. At the RAGKO endpoint, there was no difference in either IFNγ or IL-17A production by effector T cells in the colon of RAGKO and CD45RAGKO mice (Figure 4.5F).

Interestingly, T cells in the CD45RAGKO mice at the CD45RAGKO endpoint expressed higher levels of the integrin CD103, which has been suggested to be a marker specific to mucosal T cells in order to retain T cells in the gut (Suffia et al., 2005; Zhou et al., 2008) (Figure 4.6). However, there was no significant difference in CD103 expression on colon T cells between RAGKO and CD45RAGKO mice at the RAGKO endpoint suggesting that at this point when the systemic inflammation is minimal in the CD45RAGKO mice, expression of CD103 on T cells does not retain them in the colon (Figure 4.6).

Overall, these data show that T cells in the CD45RAGKO colon at the RAGKO endpoint are equivalent phenotypically and functionally to the T cells in the colon of RAGKO mice until they start to lose weight. In addition, there was no evidence to suggest that T cells are retained in the CD45RAGKO colon upon T cell transfer colitis at the RAGKO endpoint thus limiting systemic inflammation.
Figure 4.6. **CD45RAGKO mice have increased expression of CD103 on colon T cells when they reach their weight loss endpoint.** Representative flow cytometry stains of CD103 expression of TCRB+CD4+ T cells in the colon of RAGKO and 45RAGKO mice at the RAGKO endpoint and at the 45RAGKO endpoint post colitis (left). Graphs of pooled data from at least 2 experiments of the frequency and cell numbers of CD103+ colon T cells post colitis, n=8-18 (right). Data represent mean ± S.D. * p<0.05, **p<0.01, ***p<0.001.
4.2.5 Upon T Cell Transfer Colitis, CD45RAGKO Mice Have Increased Myeloid Cells in the Colon

To further analyze the inflammation in the colon, we examined the innate immune cell contribution to inflammation. There was no difference in the frequency of neutrophils and there was also no difference in the production of TNFα by these neutrophils at the RAGKO endpoint between RAGKO and CD45RAGKO mice (Figure 4.7). In contrast, myeloid cells defined as CD11c<sup>hi</sup> MHCII<sup>hi</sup> in the colon were significantly increased in frequency and cell number at the RAGKO endpoint and to a greater extent by the CD45RAGKO weight loss endpoint (Figure 4.8B). This was induced by the transferred T cells, as there was no difference in the myeloid cell populations prior to colitis induction (Figure 4.8A). There was minimal TNFα production by the myeloid cells in the gut prior to colitis but TNFα production increases upon T cell transfer induced inflammation (Figure 4.8C). This observation also suggested a feedback of inflammation induced by the T cells or T cell cytokines on bone marrow progenitors to make more monocytes in the CD45RAGKO mice in T cell transfer colitis.

While the enhanced myeloid cell population in the gut of CD45RAGKO mice at the RAGKO endpoint and even more dramatically at their own weight loss endpoint suggested that these cells are key contributors to intestinal inflammation in the CD45RAGKO mice, we wanted to assess if DCs within this myeloid cell population were selectively retained within the CD45<sup>-/-</sup> colon and therefore unable to induce appropriate effector T cells thereby preventing the spread of inflammation. At the RAGKO endpoint, there was no difference in DC frequency but the numbers of DCs in the mLNs were significantly reduced (Figure 4.9).
Figure 4.7. There is no difference in colon neutrophils or their TNFα production post colitis at the RAGKO endpoint. Representative flow cytometry stains of CD11bhiGr1hi neutrophils and their TNFα production from RAGKO and 45RAGKO colons at the RAGKO endpoint post colitis (top). Graphs of pooled data from two experiments of the colon CD11bhiGr1hi neutrophils and their TNFα frequency and cell numbers post colitis, n=7-8 (bottom). Data represent mean ± S.D.
Upon T cell transfer colitis, there are increased myeloid cells in the colon of CD45RAGKO mice. (A) Representative flow cytometry stains and graphs of cell frequency and number of pooled data from at least 5 experiments of CD11chiMHCIIhi cells in control mice, n=24-25. (B) Representative flow cytometry stains and graphs of pooled data from at least 2 experiments of cell frequency and number of CD11chiMHCIIhi cells in the colon post colitis at the RAGKO and 45RAGKO endpoint, n=7-22. (C) Representative flow cytometry ICS and graphs of pooled data from at least 2 experiments of cell frequency and number of CD11b+TNFα+ within CD11chiMHCIIhi cells in the colon pre and post colitis at the RAGKO endpoint, n=6-7. Data represent mean ± S.D. * p<0.05, **p<0.01, ***p<0.001.

Figure 4.8.
Figure 4.9. 45RAGKO mice have reduced CD11chiMHCIIhi cell numbers in the mLN at the RAGKO endpoint post colitis. Representative flow cytometry stains of CD11chiMHCIIhi cells in the mLN post colitis at the RAGKO and 45RAGKO endpoint (left). Graphs of pooled data from at least 2 experiments of CD11chiMHCIIhi frequency and cell number in the mLN post colitis, n=7-22 (right). Data represent mean ± S.D.
This suggested a defect in CD45−/− DCs to migrate from the colon to the mLN s, however, upon evaluation of CCR7 levels on CD11c+ cells in the mLN s, there was a significant increase in CCR7 expression in the CD45RAGKO mice compared to the RAGKO mice at the RAGKO endpoint (Figure 4.10). The reduction of DCs in the mLN s in the CD45RAGKO mice may therefore not be due to an inability to migrate.

4.2.6 CD45 Deficiency Results in Larger Spleens and Increased TER119+ Cells Pre and Post Colitis

To further evaluate how CD45 may be limiting systemic inflammation, we followed up on a previous observation that CD45KO and CD45RAGKO mice have enlarged spleens. Splenomegaly and anemia are often associated with colitis development in both mice and humans. Splenomegaly is thought to be due to leukocyte infiltration into the spleen but a recent report suggested that during Salmonella infection, splenomegaly was due to enhanced TER119+ cells, which include erythroblasts and mature erythrocytes, into the spleen (Jackson et al., 2010). In addition, CD71+TER119+ cells have been shown to be suppressive towards myeloid cells in the neonate and inhibit their TNFα production (Elahi et al., 2013). Since we had already observed that the spleens of CD45RAGKO mice were increased in size compared to RAGKO mice and also that CD45KO mice has larger spleens than WT mice, we sought to investigate whether this was due to altered erythropoiesis in CD45KO mice. Prior to colitis induction CD45KO mice and CD45RAGKO mice were observed to have distinctly larger spleens than WT mice and RAGKO mice (Figure 4.11A). The weights of the CD45KO spleen and the CD45RAGKO spleen were also significantly heavier than the WT and RAGKO spleen respectively. Post colitis induction at the RAGKO endpoint, there was an increase in spleen weight between the control mice and the colitis mice for both genotypes but
Figure 4.10. CD45RAGKO mice have increased CCR7+CD11chiMHCIIhi cells in the mLNs post colitis induction at the RAGKO endpoint. (A) Representative flow cytometry stains of CCR7 expression on CD11chiMHCIIhi cells in the RAGKO and 45RAGKO post colitis. (B) Graph of pooled data from 2 experiments of CCR7 frequency of RAGKO and 45RAGKO mice post colitis at the RAGKO endpoint, n=6. Data represent mean ± S.D. * p<0.05.
CD45KO mice have larger spleens and increased TER119+ cells compared to control mice pre- and post-colitis. (A) Representative images of control spleens of WT, CD45EKO, RAGKO and CD45RAGKO mice and graph of pooled data of control spleen weight from at least 2 experiments, n=6-16. (B) Graph of pooled data of RAGKO and 45RAGKO mice spleen weights at the RAGKO endpoint and at the 45RAGKO endpoint from at least 2 experiments, n=5-10. (C) Graph of pooled data from 2 experiments of TER119+ cell frequency and number in the spleen of RAGKO and 45RAGKO mice pre- and post-colitis, n=5-7. Data represent mean ± S.D. * p<0.05, **p<0.01, ***p<0.001.
CD45RAGKO mice had consistently larger spleens prior to and post inflammation (Figure 4.11B). Consistent with the larger spleens prior to and post colitis in CD45RAGKO mice compared to RAGKO mice, there was also significantly increased frequency and an increased trend for number of TER119+ cells (Figure 4.11C). Interestingly, the frequency of TER119+ cells decreased significantly by about 10% in RAGKO and CD45RAGKO upon colitis but the numbers are increased overall in colitis mice compared to control mice (Figure 4.11C). These results suggest that CD45 on erythroblasts promotes the maturation in to erythrocytes prior to and post T cell colitis. Therefore, we wanted to evaluate further the progenitors of erythrocytes in control and colitis mice and assess whether these cells could be limiting systemic inflammation in the CD45RAGKO mice in T cell transfer colitis.

4.2.7 CD45 is required for the Down-regulation of Megakaryocyte-Erythroid Progenitors in the Bone Marrow Upon Colitis

As we observed larger spleens and increased TER119+ cells in the CD45RAGKO mice pre and post colitis, we next assessed the upstream progenitors of these cells. Megakaryocyte-erythroid progenitors (MEP) are a population of highly proliferative committed progenitors that are within the Lineage\(^-\)cKit+Sca1- population, along with the granulocyte-monocyte progenitor (GMP) and the common myeloerythroid progenitor (CMP) (Na Nakorn et al., 2002). MEPs are derived from CMPs, which are derived from hematopoietic stem cells (HSCs). It has been reported that upon T cell transfer colitis, GMPs are increased at the expense of MEPs, which are reduced in order to generate more inflammatory intestinal myeloid cells (Griseri et al., 2012). In the BM, prior to colitis induction, there was no difference in the MEPs between RAGKO mice
Figure 4.12. Upon colitis induction, CD45RAGKO have increased MEPs in the bone marrow. (A) Representative flow cytometry stains of Lineage– (left-labeled with different lineage markers with two different fluorophores), cKit+Sca1- (middle), GMPs, CMPs and MEPs (right) in the bone marrow of control and colitis mice at the RAGKO endpoint. (B) Graphs of pooled data from two experiments of the frequency and cell number of MEPs, CMPs and GMPs in the bone marrow of control mice, n=6. (C) Graphs of pooled data from two experiments of the frequency and cell number of MEPs, CMPs and GMPs in the bone marrow of colitis mice at the RAGKO endpoint, n=6. Data represent mean ± S.D. * p<0.05, **p<0.01, ***p<0.001.
and CD45RAGKO mice, but CD45RAGKO mice do have decreased GMPs (Figure 4.12A,B). Upon T cell colitis induction, the GMPs are increased in both the RAGKO and CD45RAGKO mice at the RAGKO endpoint, but the MEPs are not reduced in the CD45RAGKO mice as they are in RAGKO mice (Figure 4.12A,C). Thus, in addition to abnormally increased TER119+ cells in the spleen of CD45RAGKO mice, the upstream progenitors of these cells, MEPs, are not downregulated in the BM upon colitis induction. Taken together, these data imply that in the absence of CD45, the downregulation of MEPs is compromised in T cell transfer colitis and this coupled with the already increased TER119+ population in the CD45RAGKO mice contributes to the larger spleens. It is also possible that the prevalence of these TER119+ cells may contribute to the reduced systemic inflammation seen in these mice. These results suggest that CD45 may play a role in the reduction of MEPs in colitis, which can lead to anemia and we therefore wanted to evaluate whether the populations of TER119+CD71+ cells, which have shown to be suppressive, were altered in CD45RAGKO in T cell transfer colitis.

4.2.8 CD45 Promotes Mature Erythrocyte Development and Limits Suppressive CD71+TER119+ Cells

To further characterize the effect of CD45 on erythroid maturation, we sought to investigate whether CD45RAGKO mice had enhanced frequency and/or numbers of erythrocytes and CD71+TER119+ cells erythroid progenitors. Since CD71+TER119+ cells have been shown to be suppressive in neonates, we questioned whether the increase of these cells in CD45RAGKO adult mice could correspond with a delay in systemic disease. Erythropoiesis develops in the bone marrow from MEPs to eventually become mature erythrocytes (Figure 4.13A). The development of erythrocytes goes through several stages where the final stages...
Figure 4.13. As erythrocytes mature, the expression of CD45 decreases. (A) Erythropoiesis: the maturation process of erythrocytes from MEPs. Erythrocytes along with reticulocytes and acidophilic end-stage erythroblasts are a heterogeneous population within Erythroblast C. (B) Graph of CD45+ percentage on TER119- cells (mostly leukocytes), proerythroblasts (CD71+Ter119-) and erythroblast A-C in the bone marrow and spleen of control RAGKO mice, n=4. Data represent mean ± S.D.
involve the expression of the receptors CD71 and TER119 (Figure 4.13A). As erythrocytes mature, they also down-regulate CD45 with pro-erythroblasts that are CD71+TER119- having the highest percentage of CD45. There is then a gradual reduction of CD45 from erythroblast A to C (Figure 4.13B). Erythroblast A cells are CD71+TER119+ FSC large, erythroblast B cells are CD71+TER119+ FSC small and the mature erythroblast C cells are CD71-TER119+ FSC small (Figure 4.13A).

In the spleen, prior to and post colitis, CD45RAGKO mice had an increased frequency and number of the immature erythroblast A and B (CD71+Ter119+) and a decreased frequency of the more mature erythroblast C and erythrocyte (CD71-Ter119+) population (Figure 4.14). Interestingly, in the spleen of RAGKO and CD45RAGKO mice there was a reduction of the CD71+Ter119+ population upon colitis development, but CD45RAGKO mice had a significantly increased frequency and a trend for increased numbers pre and post colitis. Consistently, like the spleen, the bone marrow of CD45RAGKO mice have increased frequency and number of CD71+Ter119+ cells prior to and post colitis (Data not shown). These results show that CD45RAGKO mice have a preexisting increase in early immature erythroblasts (A and B) and this defect is maintained upon T cell transfer colitis. Interestingly, this is when the expression of CD45 decreases upon erythroblast maturation at the erythroblast B and C stages (Figure 4.13). This implies that the downregulation of CD45 expression on early erythroblasts is needed for the proper maturation of erythrocytes and that in the absence of CD45, the signal to differentiate but not proliferate is compromised. The presence of this potentially suppressive population, together with the inability of the MEPs to downregulate in the CD45RAGKO mice in T cell colitis raises the possibility that this population may contribute to the reduction in systemic inflammation in CD45RAGKO mice. These results show that CD45 is needed for
Figure 4.14. CD45RAGKO have an accumulation of CD71+ erythroblasts pre and post colitis. (A)
Representative flow cytometry stains of CD71 and Ter119 cells (left) and varying sizes of CD71+ within that population (right), representing erythroblasts as they mature into erythrocytes in the spleen prior to and post colitis. Graphs of pooled data of two experiments of CD71+Ter119+ frequency and cell number in the spleen prior to and post colitis.
post colitis, n=5-7. (B) Pooled data from two experiments of the cell frequency and number of Erythroblast A (Ter119+CD71+FSC large), Erythroblast B (Ter119+CD71+FSC small) and Erythroblast C (Ter119+CD71-FSC small) in the spleen prior to and post colitis, n=5-7.
erythrocyte maturation and in its absence, there is an accumulation of CD71+TER119+ cells. The CD71+TER119+ cells are potentially suppressive and this could impact the outcome of intestinal colitis or systemic inflammation in these mice.

4.3 Discussion

IBD is a multifaceted disease with components of the immune system, blood and the non-hematopoietic compartment all involved in disseminating disease. The development of IBD is not just limited to intestinal inflammation as other extraintestinal complications may result (Levine and Burakoff, 2011). A common extraintestinal complication of IBD is the development of anemia that can result from EPO unresponsiveness, insufficient production of erythroid progenitors and iron malabsorption. In animal models, it has been shown that exogenous EPO can ameliorate colitis and also that CD71+ erythroid progenitors can suppress pro-inflammatory cytokine production by neonatal myeloid cells (Elahi et al., 2013; Nairz et al., 2011). In animal models of colitis the degree of weight loss is often associated with the severity of gut inflammation. However, in this report, we show that weight loss is disconnected to intestinal inflammation. We show that the absence of CD45 on innate immune cells leads to equal inflammation due to increased myeloid cells in the LP and a lack of DCs in the mLNs that may contribute to the systemic spread of disease. In addition, we show a novel role for CD45 on RBC development. Furthermore, we show an aberrant accumulation of immature RBCs in the spleen and bonemarrow, which if suppressive, may contribute to the delay in weight loss in the CD45<sup>−/−</sup> mice.

CD45 is a protein tyrosine phosphatase and is known best as a negative and positive regulator of SFKs in adaptive and innate immune cells. CD45 is only expressed on early
erythroid progenitors and its expression gradually decreases as erythroblasts mature into erythrocytes (Craig et al., 1994; Lansdorp et al., 1990). As a result of the lack of expression of CD45 on mature RBCs, a role of CD45 on erythroid development has not been considered. While it has been reported that CD45 deficient bone marrow progenitors generate increased erythroid colonies in response to EPO compared to control cells, the functional consequence of this phenomena in vivo had not been examined (Irie-Sasaki et al., 2001). Here, we define a key role for CD45 in RBC maturation. We show a novel role for CD45 in limiting CD71+ erythroblasts in the spleen and bone marrow. In addition, we show that upon inflammation induced by T cell transfer colitis, the enhanced numbers of these cells in the spleen and bone marrow of CD45RAGKO mice are maintained and MEP numbers are not decreased.

It has been shown previously that exogenous EPO can act directly on EPO-receptor positive myeloid cells and limit their production of pro-inflammatory factors such as TNFα and IL-6 (Nairz et al., 2011). EPO is also well established to act on erythroid progenitors and enhance erythrocytes in vivo (Ridley et al., 1994). CD71+ erythroblasts have also shown to be suppressive towards TNFα+ myeloid cells (Elahi et al., 2013). In addition, progenitors of erythroid cells, MEPs are downregulated upon inflammation (Griseri et al., 2012). Taken together, these various studies all suggest that the erythroid lineage and factors that promote this lineage such as EPO have an anti-inflammatory effect on the immune system. We observed enhanced CD71+ cells in the CD45RAGKO mice and since there was a corresponding delay in systemic inflammation, this raises the possibility that CD45 deficient CD71+ cells may suppress the pro-inflammatory nature of CD45 deficient myeloid cells and/or adoptively transferred effector T cells. Whether CD45 deficient CD71+ cells act on myeloid cells or effector T cells directly is still unanswered. However, given that it has been shown that CD71+ cells can
suppress TNFα production from myeloid cells, it could be that they also suppress other inflammatory mediators such as IL-12, preventing sufficient conversion of effector T cells in the CD45 deficient mice. CD45 may also regulate EPO receptor signalling on CD71+ cells. JAK2 (Neubauer et al., 1998; Parganas et al., 1998) and the SFK, Lyn (Slavova-Azmanova et al., 2014; Slavova-Azmanova et al., 2013), are kinases that are downstream of EPO receptor signalling. Since CD45 has been shown to be a JAK/STAT phosphatase and is well characterized as a regulator of Lyn, CD45 may also regulate erythroid development through either of these kinases.

Unexpectedly, the delay in weight loss did not translate into reduced local gut inflammation in the CD45RAGKO mice upon T cell colitis induction. At the time point when RAGKO mice reach their weight loss endpoint, CD45RAGKO mice have lost minimal weight but surprisingly their colon inflammation is similar to the RAGKO mice. Characterization of the cell populations in the colon revealed that the CD45RAGKO mice had equivalent T cells and increased percentage and numbers of CD11c^{hi}MHCII^{hi} myeloid cells in the gut. Interestingly, CD45 deficient bone marrow derived DCs also develop enhanced numbers over time in GM-CSF in vitro cultures (Amy Saunders, unpublished data). Furthermore, in response to TLR ligands, the pro-inflammatory cytokines, IL-6, TNFα and IL-12p40 are increased from CD45 deficient bone marrow DCs compared to wildtype BMDC (Cross et al., 2008; Piercy et al., 2006). In vivo upon disease, aspects of these results in vitro are recapitulated. Upon inflammation, CD45 deficient myeloid cells are increased in the colon, yet there is no difference in myeloid cell frequency and number between CD45RAGKO and RAGKO mice in steady state. The production of TNFα from CD45 deficient intestinal myeloid cells compared to the RAGKO mice is also similar, suggesting that CD45 deficiency in macrophages or DCs in vivo does not result in enhanced TNFα production at least.
The mLNJs in the CD45RAGKO mice upon inflammation had reduced total, T cell and DC numbers at the RAGKO endpoint. While T cells are not increased in frequency or number in the colon of CD45 deficient mice compared to RAGKO mice at the RAGKO endpoint, myeloid cells are increased in the gut compared to RAGKO mice at the RAGKO endpoint. This suggests that myeloid cells are recruited or retained within the colon of CD45RAGKO and are unable to migrate to the mLNJs accumulating in the colon. However, in contrast to this point, CCR7+ myeloid cells in the mLNJs of CD45RAGKO mice is increased implying that these CD45 deficient DCs are capable of migration from the gut to the mLNJs. Interestingly, a report by Voedisch et al. showed that upon *Salmonella* infection in mice and removal of the mLNJs resulted in increased numbers of the bacteria reaching systemic sites such as the spleen and enhanced susceptibility to the pathogen (Voedisch et al., 2009). These results suggested that the mLNJs are a barrier shielding other systemic compartments from DC bound bacterial antigens (Griffin et al., 2011; Voedisch et al., 2009). It is therefore a possibility that the enhanced numbers of CD45 deficient intestinal myeloid cells act to limit the inflammation to the gut and prevent the systemic dissemination of disease, allowing for prolonged overall survival upon T cell transfer colitis.

The expression of the integrin, CD103 is enhanced on the adoptively transferred T cells in the CD45RAGKO mice once they reach their own weight loss endpoint. There is however no difference in the CD103 expression of these T cells in the CD45RAGKO mice at the RAGKO endpoint, suggesting that T cells are not selectively retained in the CD45 deficient colon at the RAGKO endpoint; however the enhanced expression of CD103 on the T cells at the CD45RAGKO endpoint shows that these T cells may promote retention as CD103 expression on CD8+ intraepithelial cells corresponds with enhanced intestinal disease (Zhou et al., 2008). Interestingly, the expression of $\alpha_4\beta_7$ on T cells in the mLNJs of CD45RAGKO mice was
decreased at the CD45RAGKO endpoint (data not shown), suggesting that as in DSS-induced colitis, induction of gut homing molecules on CD45+ T cells is impaired in inflammation. However, unlike the DSS model, in T cell transfer colitis, numbers of T cells in the colon of CD45RAGKO mice are not reduced and these mice have comparable intestinal inflammation to RAGKO mice. This shows that impairment of gut homing molecule induction on CD45+ naïve T cells is not a contributing factor to intestinal inflammation in T cell transfer colitis.

Another possible explanation for the dichotomy between the systemic and intestinal inflammation observed in the CD45RAGKO mice is that either the CD45 deficient CD71+ erythroblasts only exert their function systemically or that intestinal CD45 deficient myeloid cells are able to overcome the suppressive nature of CD45 deficient CD71+ cells. It is clear that while the overall pathology of colon inflammation in the CD45RAGKO mice is similar to the RAGKO mice at their endpoint, the inflammatory cell populations differ. In particular, the CD45 deficient myeloid cells expand in the gut or have increased recruitment upon inflammation and as a result they may overcome any suppressive ability of EPO or CD71+ erythroblasts. In support of this, Griseri et al. reported that once inflammation develops, GMPs are increased at the expense of MEPs (Griseri et al., 2012). The enhanced GMPs are progenitors to intestinal myeloid cells that promote inflammation (Griseri et al., 2012). In the CD45RAGKO mice, MEPs are not downregulated as they are in RAGKO mice. However, in the CD45RAGKO mice, the GMPs do increase in the bone upon colitis like the RAGKO mice,. Therefore it is possible that in the CD45RAGKO mice, CD45 deficient intestinal myeloid cells compensate and overcome the suppressive ability of CD45 deficient CD71+ erythroblasts resulting in progressive intestinal inflammation. However, since CD45 deficient myeloid cells are not increased in the spleen, they
cannot overcome the suppressive effects of CD71+ cells in the spleen and blood resulting in delayed systemic wasting disease.

Overall, our results show a novel role for CD45 in promoting the maturation of erythroblasts into mature erythrocytes. Our results also show that upon inflammation induced by T cell transfer colitis, CD45 restrains the numbers of intestinal myeloid cells limiting intestinal inflammation. These results highlight the role CD45 plays in vivo on individual cell types in order to regulate inflammation. Our findings also suggest new avenues for potential therapeutics for anaemia, cachexia and intestinal inflammation.
Chapter 5: Summary and Future Directions

5.1 In DSS Colitis, CD45 Negatively Regulates Pro-Inflammatory Cytokine Production and the Expression of α4β7 on T Cells

Here, I have shown that in a model of DSS colitis, CD45 deficient T cells are not dramatically reduced in the LP of the large intestine as they are in the mLNs and spleen. It has previously been shown that in the absence of CD45, there is a block in thymocyte development resulting in a 90-95% reduction of T cells in the spleen and lymph nodes in CD45 deficient mice (Byth et al., 1996; Kishihara et al., 1993). Therefore, the comparable T cell numbers in the intestine was surprising. Corresponding with the higher than expected CD45 deficient intestinal T cells, was an increase in the percentage of T cells expressing the gut homing molecule, α4β7 in the mLNs (Figure 3.2). The dramatic increase of this gut homing molecule compared to WT mice suggests that CD45 deficient T cells have enhanced gut homing. To confirm this, future studies could compete CD45 deficient T cells and WT T cells by injecting these cells into RAG1KO mice and inducing DSS colitis. Interestingly, only α4β7 was increased on CD45 deficient T cells and not the homing molecule CCR9, suggesting that CD45 specifically regulates expression of certain adhesion molecules not chemokine receptors on T cells. Furthermore, in DSS-induced inflammation, T cell CD45 negatively regulates IFNγ, IL-17A and GM-CSF production by T cells in the intestine and the spleen. Interestingly, these results counter the perception that the T cells that do develop in CD45 deficient mice are anergic or non-functional (Kishihara et al., 1993). It was concluded that CD45KO mice are not able to mount an appropriate cytotoxic T lymphocyte response due to abrogation of footpad swelling after local infection with lymphocytic choriomeningitis virus (Kishihara et al., 1993). This may be due to
insufficient CD45KO T cell numbers but the T cells may intrinsically be able to make sufficient or exacerbated pro-inflammatory cytokines, which was not assessed in this study. The T cells that do develop in the CD45 deficient mice are shown to have a more activated phenotype with enhanced expression of the activation markers, CD44 and LFA-1 (Kong et al., 1995), but it is still unclear whether TCR signalling is actually impaired in the T cells that do develop in the CD45 deficient mice and why these T cells are able to overcome the thymocyte developmental defect.

5.2 In the Absence of an Adaptive Immune System, CD45 Positively Regulates GM-CSF and IL-22 Production by ILCs and Retinoic Acid Production by CD103+ DCs

There was no difference in DSS colitis severity between CD45RAGKO and RAGKO mice. However, there were notable differences in cell populations and cytokine production in the intestine. CD45 deficient ILCs had lower IL-22 and GM-CSF production, CD45 deficient intestinal myeloid cells were decreased and there was also lower RA production by CD45 deficient CD103+ DCs in the mLNs. When CD45 sufficient polyclonal T cells were adoptively transferred into CD45RAGKO and RAGKO mice, CD45RAGKO mice had less severe colitis as a result of impaired gut homing of effector T cells. I hypothesized that the impaired homing of effector T cells was a result of lower GM-CSF in the CD45 deficient gut, resulting in lower RA production. Previous studies have demonstrated that in an inflammatory and a homeostatic environment, RA induced homing molecules on effector T cells and Tregs (Coombes et al., 2007; Hall et al., 2011). Therefore, I predicted that by providing exogenous GM-CSF or RA, the reduction of effector T cells in the CD45 deficient gut would be increased and indeed, this was what was observed. These results expand the recent findings on the communication between IL-
1-, RA- and GM-CSF-producing cells in the intestine and their influence on effector or regulatory T cells. Mortha et al. demonstrated that IL-1β produced by intestinal macrophages induces GM-CSF production by ILCs, which in turn maintained CD103+ DCs and enabled them to produce RA, thus inducing sufficient Tregs to maintain oral tolerance (Mortha et al., 2014). Conversely, Basu et al. showed that IL-1 abrogated RA-mediated suppression of Th17 cells, resulting in protection during Citrobacter rodentium infection (Basu et al., 2015). In addition, Basu et al. demonstrated that IL-1 is a potent inhibitor of RA-driven Treg differentiation but these findings also contradict the findings by Mortha et al. that in steady state, IL-1 indirectly promotes RA-Treg differentiation through GM-CSF and by Hall et al. that RA receptor α deficient mice have impaired Th17 differentiation (Basu et al., 2015; Hall et al., 2011; Mortha et al., 2014). In addition to the findings in this project, it is clear that the dominant influence of IL-1, GM-CSF and RA on helper T cells is dependent on the inflammatory situation. It may be that in steady-state, when there are copious amounts of IL-10 in the intestinal environment, IL-1 induces GM-CSF by ILCs and the eventual induction of Tregs. In inflammation, when IL-10 is overwhelmed by other pro-inflammatory mediators such as TNFα, IL-23 and IL-6, IL-1 may counter RA-Treg differentiation and promote induction of Th17. Furthermore, the factors that determine whether Treg differentiation is inhibited and Th17 promoted and/or whether RA induces effector T cell homing may also depend on the type of inflammatory situation. Here we showed CD45 regulates GM-CSF production promoting increased RA and ultimately effector T cell homing to the gut. However, whether this is through IL-1 signaling in ILCs is not yet known. Therefore, it will be imperative to determine whether IL-1 production is altered or whether another inflammatory cytokine such as IL-23 regulates RA production in the CD45 deficient gut. Another possibility is whether CD45 regulates MyD88-IRAK signaling in the IL-1 signaling
pathway, as it does TLR dependent signaling, leading to altered cytokine production. Alternatively, CD45 may also regulate the signaling pathway leading to GM-CSF production or CD45 may regulate the production of IL-1 by myeloid cells leading to impaired GM-CSF production. Determining how CD45 regulates cytokine production will illuminate further how cells communicate with each other in a complex cytokine environment upon inflammation and why certain inflammatory situations yield distinct pathways of effector T cell function.

5.3 CD45 on Innate Immune Cells Potentiates Systemic Inflammation in T Cell Transfer Colitis

Systemic Inflammation in CD45RAGKO mice is attenuated compared to RAGKO mice upon the transfer of naïve CD4+CD25−CD45RBhigh T cells. These results mimic the delay in systemic weight loss in the transfer of polyclonal CD45+ T cells into CD45RAGKO mice and DSS colitis. However, there is one pointed difference; in the T cell transfer model, intestinal inflammation is similar between RAGKO and CD45RAGKO mice. This dichotomy with reduced systemic inflammation but equivalent intestinal inflammation in the CD45RAGKO mice compared to RAGKO mice could be explained by the enhanced CD71+ erythroblasts in the CD45RAGKO mice. CD71+ erythroblasts and EPO, the hormone that induces the differentiation of these cells, have been shown to be suppress TNFα production in several studies using colitis models and bacterial infections (Jackson et al., 2010; Nairz et al., 2011). Interestingly, these reports also showed that EPO or CD71+ cells suppressed intestinal inflammation, which differs from the results of this project. The enhanced number of CD45 deficient inflammatory myeloid cells that may overcome the suppressive ability of CD45 deficient erythroblasts may account for these contradictory results. In fact, Jackson et al. showed that even though neutralization of EPO
substantially decreased the immature CD71+ RBCs, there was only a modest increase in control of host infection (Jackson et al., 2010). While the role of CD45 deficient CD71+ erythroblasts were not taken into account in the DSS model of colitis, it may be possible that these immature RBCs are suppressive in CD45RAGKO mice upon DSS, especially upon the transfer of CD45+ polyclonal T cells. Nevertheless, whatever potential suppressive role CD45 deficient CD71+ erythroblasts do play is completely overcome by the addition of GM-CSF or RA.

To definitively show that CD45 deficient CD71+ erythroblasts are suppressive, an assay to inject CD45 deficient CD71+ erythroblasts into RAGKO mice upon T cell transfer colitis to ameliorate colitis in these mice or depletion of CD71+ cells in CD45RAGKO upon T cell transfer colitis to worsen the systemic inflammation in these mice will clarify these questions. Alternatively, co-culture of CD71+ erythroblasts with TLR-stimulated BMDCs to assess if the addition of CD71+ cells suppress pro-inflammatory cytokine production by the BMDCs may also give insights into the suppressive nature of these immature RBCs. Furthermore, it will also be imperative to determine how CD71+ erythroblasts are suppressive. Nairz et al. demonstrated that EPO is suppressive by its binding of the EPO receptor on inflammatory myeloid cells to diminish pro-inflammatory cytokine production, but it is unclear how CD71+ erythroblasts mediate suppression (Nairz et al., 2011). A possible explanation is that CD71+ cells secrete anti-inflammatory mediators such as IL-10 or TGFβ. Moreover, in the T cell transfer colitis model, it is also unclear whether CD71+ erythroblasts mediate their suppression directly on myeloid cells or T cells. While Elahi et al. demonstrated in co-culture experiments that CD71+ erythroblasts can suppress TNFα production from intestinal CD11b+ cells from neonates (Elahi et al., 2013), it is also possible that CD71+ cells suppress myeloid cells in vivo in CD45RAGKO upon T cell transfer colitis CD71+ erythroblasts may mediate suppression of multiple pro-inflammatory
cytokines by DCs or macrophages systemically that make it an unfavorable environment for the conversion of naïve T cells into effector T cells. It is also a possibility that CD71+ erythroblasts directly limit effector T cell proliferation by several ways such as limiting IL-7 or IL-2 in the spleen.

Notably, the percentage of cells expressing CD45 steadily decreases as erythrocytes mature (Figure 4.13). Pro-erythroblasts are CD71+TER119- and express CD45 as all other TER119- leukocytes. As TER119 is expressed on erythroblasts, the percentage of cells that CD45+ decreases dramatically in the bone marrow and the spleen. In CD45 deficient mice, the accumulation of cells is at the erythroblast A and B stage, so as CD45 expression decreases. It is evident that the downregulation of CD45 is necessary for the downregulation of immature erythroblasts but how this is regulated is not clear.

5.4 Perspectives

Herein, novel and unexpected roles for CD45 in various immune cells have been identified. I have shown that CD45 on T cells, ILCs, DCs and immature RBCs regulates the production of various cytokines and factors that mediates immune communication. The role of CD45 in vivo upon intestinal inflammation was also established in the projects here and CD45 was shown to regulate pro-inflammatory cytokine production by T cells, ILCs and myeloid cells and the maturation of immature RBCs. In the absence of CD45, the presence or absence of an adaptive immune system determined disease severity. This highlights the bi-directional communication between the adaptive and innate immune system. In the absence of T and B cells, CD45 deficiency in the innate immune system results in impaired production of RA and GM-CSF but in the presence of adaptive immune cells, this deficiency is overcome. These results
show that CD45 regulates the production of various cytokines from effector Th1, Th17, ILC1 or ILC3 cells. However, still nothing is known about the impact of CD45 on type 2 immunity. As a result, it is also likely that CD45 regulates the production of Th2 and ILC2 cytokines. Furthermore, the CD45 deficient T cells at other mucosal sites such as the lung and skin have not also been analyzed. Therefore, it is of interest to find the roles of CD45 during type 2 inflammation such as helminth infection or allergy.

Finally, the projects here also reveal new avenues of therapeutics for intestinal diseases. By targeting CD45, several cytokines may be affected at once providing a more complete treatment for disease. Furthermore, these results reveal the complexity of the intestinal immune system and how easily the proper communication between immune cells can be broken down or exacerbated. The absence of CD45 does not just affect the function of one cell-type but causes a domino effect on multiple cells and their functions.
Bibliography


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Appendix A The Absence of CD45 on the Innate Immune System Ameliorates Anti-CD40 Induced Colitis

A.1 Introduction and Rationale

The interaction of CD40 on APCs with CD40L (CD154) on T cells has been shown to be important for the mediation of intestinal inflammation (Liu et al., 2000; Polese et al., 2003). Blockade of CD40-CD40L interactions inhibits colitis development and ameliorates established disease as well (Cong et al., 2000; De Jong et al., 2000; Liu et al., 2000). It has recently been shown that the addition of an agonistic antibody to mice deficient of T and B cells induces acute colitis (Uhlig et al., 2006b). This particular animal model of colitis is characterized by an increase in intestinal myeloid cells secreting the pro-inflammatory cytokine, IL-23. IL-23 has been shown to act on RORγt+ ILC3s and induce their production of IL-17A and IFNγ (Buonocore et al., 2010; Uhlig et al., 2006b).

CD45+/− BMDCs have enhanced pro-inflammatory IL-6, IL-12p40 and TNFα cytokine production in response to both TLR2 and TLR9 ligands (Cross et al., 2008). CD45 has been implicated in modulating CD40 signaling in APCs (Huntington et al., 2006; Tan et al., 2000a). CD40 ligation activates protein tyrosine kinases including Lyn, Fyn, Syk and Jak3 (Hanissian and Geha, 1997; Ren et al., 1994). CD40 ligation also induces the activation of phosphoinositide-3 kinase (PI-3 kinase) and phospholipase Cγ2 (Ren et al., 1994). CD45 is well-known for its role in regulating SFK activity and since SFKs are involved in CD40 signaling, it is possible that CD45 also directly or indirectly affects CD40 signaling. The role of CD45 on CD40 signaling has been assessed in vitro; CD45 is able to inhibit CD40L/CD40 mediated-microglia activation.
Furthermore, co-treatment of microglia with CD40L, in the presence of CD45 activating antibody, results in significant inhibition of microglial TNFα production through inhibition of p44/42 MAPK activity (Tan et al., 2000a). Taken together, these results suggest that CD45 on APCs may regulate CD40 signaling in vivo and inhibit pro-inflammatory cytokine production. Therefore, in this study, I hypothesized that colitis will be more severe in CD45 deficient mice induced with colitis by the injection of an agonistic anti-CD40 antibody compared to control mice due to enhanced pro-inflammatory cytokine production by CD45 deficient APCs.

A.2 Methods

In Vivo Antibody Treatment

150µg of ant-CD40 IgG2a monoclonal antibody FGK45 was injected i.p. into sex- and age-matched mice on day 0. After antibody injection, mice were monitored daily and euthanized upon full recovery, on day 7 or day 10 of the experiment as stated.

A.3 Results

CD45 deficient mice develop less severe disease upon anti-CD40 induced colitis

To investigate the role of CD45 on CD40 signaling on APCs in vivo, I injected anti-CD40 antibody into RAGKO and CD45RAGKO mice. Compared to RAGKO mice, CD45RAGKO mice had a delay in weight loss and weight recovery upon anti-CD40 injection suggesting less severe colitis (Figure A1). To support their ameliorated disease, CD45RAGKO mice also had less total cell number in the colon on day 7 post colitis induction (Figure A1). These results contradict my hypothesis and suggest in vivo, CD45 may negatively regulate
cytokine production via CD40 signaling. Since the injection of anti-CD40 antibodies has previously been shown to cause enhanced myeloid cells in the colon, I next assessed whether there were any phenotypic changes in this population upon colitis induction. On day 7 post colitis induction, CD45RAGKO mice have reduced frequency and number of CD11c^{hi}MHCII^{hi} cells in the colon compared to RAGKO mice (Figure A2A and A2B). Additionally, within the CD11c^{hi}MHCII^{hi} population, CD45RAGKO mice have reduced frequency and numbers of CD103- cells and an increase in frequency of CD103+ cells (Figure A2C and A2D). Overall, on day 7 post colitis induction, CD45RAGKO mice have significantly increased frequency and numbers of CD103+CD11b- myeloid cells, no difference in the CD103+CD11b+ migratory myeloid population and a significant reduction in the CD103-CD11b+ myeloid population (Figure A2C and A2D).
Figure A.1. CD45RAGKO mice develop less severe anti-CD40 mediated colitis. (A) Representative experiment from 3 of weight loss and recovery upon the i.p. injection of 150μg of anti-CD40 agonistic antibody in RAGKO and CD45RAGKO mice. (B) Representative experiment from 3 of total cell number in the colon of RAGKO and CD45RAGKO mice prior to and post colitis, n=3-9. Data represent mean ± S.D. * p<0.05, **p<0.01.
Figure A.2. CD45RAGKO mice have reduced CD11c\textsuperscript{hi}MHCII\textsuperscript{hi} cells in the colon post colitis. (A) Representative flow cytometry labeling of CD11c\textsuperscript{hi}MHCII\textsuperscript{hi} cells in the colon on day 7 post colitis and the percentage of CD103 and CD11b within this population in RAGKO and CD45RAGKO mice. (B) Representative experiment from 3 of the frequency and cell number CD11c\textsuperscript{hi}MHCII\textsuperscript{hi} cells in the colon on day 7 post colitis in RAGKO and CD45RAGKO mice, n=3-4. (C) Representative experiment from 3 of the percentage and number of CD103+ and CD103- cells within the CD11c\textsuperscript{hi}MHCII\textsuperscript{hi} cells in the colon on day 7 post colitis in RAGKO and CD45RAGKO mice, n=3-5. (D) Representative experiment from 3 of the percentage and number of CD11b+ and CD11b- within both the CD103+CD11c\textsuperscript{hi}MHCII\textsuperscript{hi} cells and the CD103-CD11c\textsuperscript{hi}MHCII\textsuperscript{hi} cells in the colon on day 7 post colitis in RAGKO and CD45RAGKO mice, n=3-5. Data represent mean ± S.D. * p<0.05, **p<0.01, ***p<0.001.
A.4 Discussion

These results show that in the absence of CD45 on the innate immune system, the induction of colitis by anti-CD40 is ameliorated. Unlike RAGKO mice, CD45RAGKO mice have reduced frequency and numbers of colon CD11c\textsuperscript{hi}MHCII\textsuperscript{hi} cells. This may be due to impaired signaling via CD40 on CD45 deficient CD11c\textsuperscript{hi}MHCII\textsuperscript{hi} cells in the colon and thus lower expansion or proliferation of these cells and as a result reduced colitis development and enhanced recovery. Alternatively, CD40 signaling in CD45 deficient colon myeloid cells may result in lower pro-inflammatory cytokine production such as IL-12, IL-23, IL-1β and/or TNFα. The impaired production of these cytokines may create an environment more favorable to CD103+ myeloid cells versus CD11b+ inflammatory myeloid cells in the CD45RAGKO mice compared to the RAGKO mice. In addition, my previous work in Chapter 3 shows that impaired GM-CSF production by ILCs impairs retinoic acid production by DCs and correlates with a reduction in CD45 deficient myeloid cells in the colon (Figure 3.6 and 3.8). It is also possible that impaired CD40 signaling in CD45 deficient colon myeloid cells leads to impaired GM-CSF production by CD45 deficient ILCs and a feedback loop that prevents further expansion of CD45 deficient myeloid cells in the colon.

Future studies should assess the cytokine environment in the CD45RAGKO mice post anti-CD40 colitis development to determine whether any pro-inflammatory cytokines are altered leading to ameliorated disease. Future studies should also assess the CD45 deficient ILCs to determine whether the population itself and/or the cytokine production is also altered. Further assessment of the phosphorylation levels of the SFKs or Jak3 may also determine whether CD45 is regulating the SFKs or members on the JAK/STAT pathway upon CD40 signaling. These
studies may give insight as to why CD45 deficient myeloid cells are reduced upon both anti-
CD40 and DSS induced colitis and whether this pathway ultimately leads to lower RA
production by CD45 deficient myeloid cells and/or lower GM-CSF production by CD45
deficient ILCs upon intestinal inflammation.