Role of different dendritic cell subsets in vivo

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NANYANG
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ROLE OF DIFFERENT DENDRITIC CELL SUBSETS *IN VIVO*

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ABBREVIATIONS

APCs	Antigen presenting cells
APC	allophycocyanin (fluorophore)
BAC	bacterial artificial chromosome
cDCs	Conventional dendritic cells
CDP	Common dendritic cell progenitor
CD	Crohn's disease
DMEM	Dulbecco's modified Eagle medium
DC	Dendritic cells
DSS	Dextran sodium sulphate
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
ECM	Extracellular matrix
FACS	Fluorescent-activated cell sorter
FITC	Fluorescein isothiocyanate
FCS	Fetal calf serum
GMP	Granulocyte/macrophage progenitor
GFP	Green fluorescence protein
ILCs	Innate lymphoid cells
IBDs	Inflammatory bowel diseases
IEC	Intestinal epithelial cells
IELs	Intra-epithelial leukocytes
i.p	Intra-peritoneal
IMDM	Isocove's modified Dulbecco's medium
MCFA	Medium-chain-length fatty acids
MLN	Mesenteric lymph nodes
MDP	Macrophage-dendritic cell progenitor
PAMPs	Pattern-associated molecular pattern
pDCs	Plasmacytoid dendritic cells
PP	Peyer's patches
PBS	Phosphate buffered saline

PE	Phycoerythrin
PFA	Paraformaldehyde
TLRs	Toll-like receptors
T _{RM} cells	Tissue resident memory
TED	Trans-epithelial dendrites
UC	Ulcerative colitis
WT	Wild type

SUMMARY

Intestinal dendritic cells (DCs) are involved both in maintenance of gut tolerance as well as in regulating innate and adaptive immune responses. However, the exact pro- and anti-inflammatory roles of different mucosal dendritic cells subpopulations in the gut are not yet fully elucidated. In order to begin detangling the specific roles of intestinal DC subsets, we have generated new transgenic mouse strains; Clec9A-DTR mice and Clec4a4-DTR mice that allow us to efficiently *in vivo* ablate CD11b⁻CD103⁺ or CD11b⁺CD103⁺ colonic DC subsets respectively. We subjected both mouse strains to a DSS-induced acute colitis model to study the consequence of colonic inflammation in the absence of either colonic DC subset. We observed in comparison to WT, the ablation of CD11b⁻CD103⁺ DCs in Clec9A-DTR mice but not CD11b⁺CD103⁺ DCs in Clec4a4-DTR mice caused a dramatic exacerbation of DSS-induced intestinal inflammation. Exacerbated clinical symptoms of colitis observed in Clec9A-DTR mice include rapid weight loss, higher presence of fecal occult blood, massive colonic neutrophil and monocytes infiltration, increased intestinal epithelial permeability and erosion of mucosa. On the other hand, in comparison to WT mice, Clec4a4-DTR mice were observed to be resistant to DSS-induced colitis and did not exhibit the severe symptoms as observed in Clec9A-DTR mice. Microarray analysis of intestinal epithelial cells obtained from day 4 DSS-treated Clec9A revealed a down-regulation in a series of IFN- γ -induced genes such as *Ido1*, *Il-18bp* and MHCII-related genes which have been shown to be important in controlling colonic inflammation. Indeed, IFN- γ production by CD8⁺ IEL, and both CD8⁺ and CD4⁺ LP T cells were markedly reduced in the absence of LP CD103⁺CD11b⁻ DCs. In addition, IECs from DSS-treated Clec9A-DTR also showed compromised expression of anti-microbial peptides *Reg3 β* , *Reg3 γ* , *S100a8/9* and MUC2 which are critical for maintaining epithelial barrier function. In contrast, intestinal epithelial cells from colitis-resistant Clec4a4-DTR mice showed a dramatic increase in the expression of anti-microbial peptides *Reg3 β* , *Reg3 γ* and *S100a8/9*, as well as the depletion in IL-22 neutralizing protein *Il22bp*, both in steady state and day 4 DSS-treatment. Hence our data clearly highlights for the first time, the important functions of CD103⁺CD11b⁻ and CD103⁺CD11b⁺ colonic DCs in regulating intestinal immune homeostasis through modulating epithelial barrier function. In this thesis we demonstrate both

CD103⁺CD11b⁻ and CD103⁺CD11b⁺ colonic DCs differentially regulate intestinal inflammation and imbalance in the ratio of either DC subset impacts intestinal immune homeostasis.

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1. INTRODUCTION

1.1 Dendritic Cells (DCs)

Dendritic cells (DCs) are hematopoietic cells belonging to the antigen-presenting cell (APC) family. These rare immune cells are scattered in both lymphoid and non-lymphoid organs throughout the body serving as coordinators of the immune system. DCs express a variety of pattern recognition receptors, such as Toll-like receptors (TLRs) and NOD proteins which enable them to recognize pathogen associated molecular patterns (PAMPs) that are typically expressed on pathogens. The bone marrow (BM) is a source of both myeloid and lymphoid DC precursors from where they emerge into the systemic circulation and travel to the lymphoid and non-lymphoid tissues throughout the body. DCs residing in tissues manifest an immature state, in which they constantly sample the local environment; upon receipt of an activation signal, they become activated and migrate to draining lymph nodes (LNs), where they express high surface levels of antigen-loaded major histocompatibility complex (MHC) molecules along with other co-stimulatory signals and cell-cell adhesion molecules to activate a naïve T cell response. The activated DCs also secrete a variety of cytokines to initiate an appropriate T cell response to eliminate the particular pathogen. Hence DCs play a central role during orchestration of immune defense through regulating other immune cells.

The study of DCs has been a slow progress since the discovery of Langerhans cells in 1868, because they have been notoriously known to be difficult to isolate from tissues and the lack of specific markers to distinguish them from monocytes/macrophages. Until the 1990s, where techniques to isolate and generate DCs from blood and bone marrow (BM) were developed, led to an explosive growth in the study of DCs [1-3]. The early pioneers of dendritic cell research by Steinman *et al* observed highly adherent spleen cells in culture dishes, which were found to have large contorted nucleus and constantly forming cytoplasmic branches resembling dendrites. Hence the term “dendritic cells” was coined from this fortuitous observation [1]. Although the role of DCs as potent APCs has been known since their discovery more than three decades ago, it was only found recently that DCs other than playing a role in inducing

pro-inflammatory response, were also required in establishing tolerance to self- and non-pathogenic foreign antigens [4]. Interaction of naïve T cells with immature resting DCs results in antigen-specific T cell tolerance, whereas mature DCs induce T cell priming. Considering the dual capabilities of DCs, they are pivotal in setting a fine balance between protective and pathologic immune response.

1.1.1 Development of DCs from Hematopoietic Precursors

DCs are classified into two main subsets that include classical DCs (cDCs) and plasmacytoid DCs (pDCs) [5]. cDCs form a heterogeneous population of hematopoietic cells that reside in all lymphoid, interface and connective tissues. Progress has been made in recent years to understand the origin and transcriptional programs that controls DC differentiation in vivo from hematopoietic precursors. This allows the study of homogeneous lymphoid and non-lymphoid DC cell populations that were derived from committed precursors along a distinct differentiation program, enabling categorization of DCs based on developmental regulation (Figure 1.1).

Figure 1.1. Origin of Lymphoid and Non-lymphoid Tissue-Resident DCs in Mice.

DCs and macrophages of the lymphoid and non-lymphoid tissues arise from a common hematopoietic precursor known as MDP which gives rise to either CDP or monocytes. CDP further differentiates into pre-DCs which may give rise to either lymphoid tissue $CD45^+CD11c^+MHCII^+CD11b^+CD4^+CD8\alpha^+$ DCs or peripheral tissue $CD11b^+CD103^+$ DCs. Monocytes derived from MDP, differentiate into either peripheral tissue macrophages or $CD11b^+CD103^-$ DCs. Transcription factors (black), and cytokine receptors (red) needed for each DC subset were illustrated above. Figure obtained with permission from [6].

Development of DCs and macrophages begins from the differentiation of Granulocyte/macrophage progenitor (GMP) to macrophage-dendritic cell progenitor (MDP) in the bone marrow. MDP has lost the ability to differentiate into granulocytes and can only give rise to monocytes and DC restricted precursor also known as common DC precursors (CDPs) [6]. CDPs can either give rise to plasmacytoid DCs (pDCs) or pre-DCs that circulate in the blood. Pre-DCs further differentiate into various cDC subsets upon entering lymphoid and peripheral tissues. In lymphoid tissues, pre-DCs give rise into either $CD4^-CD8a^+$ and $CD4^+CD8a^-$ DCs while in non-lymphoid tissues they give rise to $CD103^+CD11b^-$, $CD103^-CD11b^+$ DCs, and the gut-unique $CD103^+CD11b^+$ DCs [6]. Flt3 (Fms-like tyrosine kinase-3) controls myeloid precursor commitment to the DC lineage as well as the differentiation of mature DCs in tissue [7]. The transcription factors BATF3 (Basic Leucine Zipper Transcription Factor, ATF-Like 3), ID2 (Inhibitor of DNA Binding-2) and IRF8 (Interferon regulatory factor-8) control the differentiation of lymphoid tissue $CD8^+$ DC and non-lymphoid tissues $CD103^+CD11b^-$ DC, but do not control $CD11b^+$ DC differentiation in lymphoid and non-lymphoid tissues [6].

1.2 The Gut Immune System

The digestive tract has a surface area nearly 200 times greater than that of the skin. Being an important port of entry for microorganisms, the gut must be protected by effective immune responses. However, immune responses must be well-coordinated to prevent damage of gut tissues in response to harmless commensals and foreign antigens to which the gut is continuously exposed. It has been shown that intestinal DCs are pivotal in initiating, stimulating and regulating antigen-specific T cell responses as well as maintenance of tolerance towards commensal microflora [9, 10]. Here we illustrate the different subsets of intestinal dendritic cells and macrophages of the gut immune system.

1.2.1 Dendritic Cell Subsets and Macrophages of the Gut Immune System.

1.2.1.1 Intestinal DCs

Mouse gastrointestinal DCs are found widely distributed in intestinal lamina propria, and other organized lymphoid organs which includes: Peyer's patches (PP), colonic isolated lymphoid follicles (ILFs) and mesenteric lymph nodes (MLNs). Recent studies have identified various markers expressed uniquely on DCs hence enabling their classification into different subsets. These markers include the integrins CD11c (α X), CD103 (α E β 7) and CD11b (α M). CD103⁺ DCs can be subdivided based on differing expression of CD11b, hence they can be classified as CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs. CD103⁺CD11b⁻ DCs are found to predominate the colonic lamina propria and in lymphoid tissues such as ILFs, PPs and MLNs [16, 17, 22, 218]. Previous assumptions which denotes CD103⁺CD11b⁻ DCs develop only from lymphoid organs were debunked with new findings that ROR γ t^{-/-} mice lacking PPs and ILFs still retained this DC subset in the LP [219]. The development of CD103⁺CD11b⁻ DCs is dependent on the transcription factor BATF3, thereby they form the equivalent mucosal counterparts of classical spleen CD8⁺ DCs [220]. In addition, both spleen CD8⁺ DCs and CD103⁺CD11b⁻ DCs derive from a common pre-DC precursor [16, 18]. In contrast, CD103⁺CD11b⁺ DCs predominate the small intestine lamina propria. This subset of DC exhibit classical DC activities such as migration to mesenteric lymph

nodes, and presenting acquired antigens from lamina propria to naïve T cells [16, 29, 221].

1.2.1.2 Uptake of antigens by CD103⁺ DCs

CD103⁺ DCs reside deep in the intestinal LP and also in close association with intestinal epithelium, where they act as sentinels of the immune system performing functions such as: sensing inflammation, sampling of luminal antigens and migrating to MLNs – the site of interaction with T cells [16, 29]. In the small intestines, CD103⁺CD11b⁺ DCs reside deep within the LP [16, 29] hence were difficult to explain their capability of acquiring antigens from the lumen, unlike IEC-associated CX3CR1^{hi} macrophages that were found to have easy access to the epithelium. However, recent studies using advanced microscopy techniques such as two-photon microscopy have identified a small population of small intestinal CD103⁺ DCs at steady-state having the capacity to migrate from the deep LP to the epithelium, where they form thick transepithelial dendrites enabling the sampling of luminal *Salmonella* [9]. These antigen-sampling DCs were also observed to be more motile than CX3CR1^{hi} macrophages. Hence, DCs provide an additional route of entry for luminal antigens other than CX3CR1^{hi} macrophages.

Upon the uptake of antigens, CD103⁺ DCs constitutively migrate to the mesenteric lymph nodes during non-inflammatory environment unlike CX3CR1^{hi} macrophages [16, 29]. The constitutive migration of CD103⁺ DCs to MLNs in steady state is independent of TLRs and commensals hence may be an intrinsic property of these DCs as determined by their differentiation program [223]. The migration of CD103⁺ DCs is dependent on CCR7 since CCR7 deficient DCs failed to migrate to MLN [25].

1.2.1.3 CD103⁺DCs establish intestinal tolerance through communication with T cells

After acquiring antigens in steady state condition, mainly from harmless commensals and diet, CD103⁺ DCs migrate to mesenteric lymph nodes during non-inflammatory conditions where they interact with naïve T cells to induce intestinal tolerance. CD103⁺ DCs are endowed with tolerance-inducing mechanisms exclusive to the gut mucosa which are key to maintenance of intestinal homeostasis. One of these mechanisms include the expression of transforming growth factor-β (TGF-β) and

retinoic acid (RA) by LP and MLN CD103⁺ DCs which are potent mediators for priming naïve T cells into Foxp3⁺ T reg cells [19, 26] and differentiation of B-cells to IgA-producing plasma cells [224, 225]. Crucial to the gut-homing ability of primed Foxp3⁺ T reg cells, CD103⁺ DCs imprint these T cells, via RA-dependent signaling, with the gut homing receptor CCR9 and $\alpha 4\beta 9$ which enables them to traffic into peripheral gut tissues from circulation [29, 226, 227]. CD103⁺ DCs express retinaldehyde dehydrogenase (RALDH2) which converts retinol to RA, which has been described above to support the tolerogenic functions of CD103⁺ DCs. Lastly, CD103⁺ DCs also express indoleamine 2,3 dioxygenase 1 (IDO1), which has been shown to arrest effector T cell development and promote Treg cell generation, through conversion of tryptophan resulting in accumulation of its product kynurenines in the mucosa [228]. The tolerogenic properties of CD103⁺ DCs is prevalent in homeostatic conditions. IECs have been shown to be important in ‘educating’ CD103⁺ DCs to acquire this role [34], and the mechanisms of how IECs perform this role is described in section 1.2.4.3.

1.2.1.4 Dendritic cells of the Gut-Associated Lymphoid Tissue (GALT)

The GALT consists of the MLN, Peyer’s patches, small intestine isolated lymphoid follicles and colonic tertiary lymphoid tissue. The mesenteric lymph node mononuclear phagocytes can be divided into either resident or migratory cells. The migratory cells were derived from the lamina propria while the resident mononuclear phagocytes are from blood-derived precursors. The CD11c^{hi} resident classical DCs major subsets includes CD11b⁻ CD8 α ⁺, CD11b⁻ CD8 α ⁻ and CD11b⁺ CD8 α ⁻ cells. Gut tolerance to commensals and food antigen is mediated by the migratory CD103⁺CD11b⁺ DCs from the lamina propria. Carriage of antigens to the mesenteric lymph nodes is mediated through the expression of CCR7 in these cells after antigen exposure, leading to activation of T cells towards regulatory of effector T cell fates [19]. The CX3CR1⁺ DCs residing in the mesenteric lymph nodes were derived exclusively from blood-derived Ly6C^{hi} monocytes [18] and not contributed by migrants from the lamina propria.

Peyer’s patches acquire antigens from the intestinal lumen through specialized epithelial microfold cells also known as M cells [21]. These lymphoid organs lack afferent lymphatics and are structurally similar to lymph nodes. Subsets of DCs in the

Peyer's patches includes CD11b⁺ DCs (subepithelial dome (SED)), CD8α⁺ DCs (T cell-rich interfollicular regions) and CD11b⁻ CD8α⁻ DCs (sub-epithelial dome, interfollicular regions and follicular-associated-epithelial area) [22]. The CD11b⁺ Peyer's patch DC subset primes T_H2 cell generation while other subsets prime mainly T_H1-type polarization [22]. Most importantly, CD11b⁺ Peyer's patch DCs are needed for class-switching of naïve B cells towards IgA production through the unique expression of IL-6 [22]. T cells primed by Peyer's patch DCs were imprinted with α4β7 integrin hence are able to home in gut tissues from circulation. Similar to the MLNs, SED resident Peyer's patches CX3CR1⁺ CD11b⁺ DCs were derived from Ly6C^{hi} monocytes [23].

1.2.1.5 Intestinal macrophages.

Intestinal macrophages form the biggest pool of total body macrophages and their sheer numbers outnumber that of DCs [232]. The characterization of these intestinal macrophages rely on the surface expression of integrins such as CD11c and CD11b, and F4/80 [232]. Other than these molecules, intestinal macrophages also express CD64 (FCγR1) [234] and the chemokine receptor CX3CR1 [233] which both not expressed by CD103⁺ DCs. CX3CR1 macrophages are derived from Ly6C⁺ blood monocytes [16, 18], and their ability to home into the gut is dependent on the chemokine receptor CCR2, which recognizes CCL1 uniquely expressed by the gut mucosa in response to low-grade tonic inflammation [235]. Moreover, these stable homeostatic tissue-resident immune cells exhibit a half-life of 3 weeks, which is atypical of tissue macrophages [221].

CX3CR1^{hi} macrophages have been reported to extend transepithelial dendrites (TEDs) towards the luminal space to probably sample commensals [208]. The formation of these TEDs in terminal ileum were specific to these CX3CR1⁺ macrophages and dependent on interaction between CX3CR1 and its IEC-expressed CX3CL1/Fractalkine [236]. Limited studies have demonstrated the functional significance of the formation of these TEDs leading to sampling of bacteria from the lumen. Of note, the intact epithelium itself has evolved some mechanisms of allowing luminal antigens access through the barrier, such as through specialized M-cells and antigen-channeling goblet cells [237]. In addition, as mentioned previously, CD103⁺CD11b⁺ DCs were capable of migrating to the intact epithelium to sample

luminal bacteria, hence do not require support from CX3CR1 macrophages to acquire luminal antigens [9]. However, it has been demonstrated that these CX3CR1⁺ macrophages provide an alternative Myd88-independent route for entry of non-invasive *Salmonella typhimurium* mutants [238], hence CX3CR1⁺ macrophages directly contribute to the sampling of luminal antigens along with other pathways of antigen entry. Deficiency in CX3CR1 leads to the inability of macrophages to form TEDs [210]. However this does not result in defective entry of luminal antigens to the LP and subsequent priming of T cells in MLNs. These mice however failed to establish oral tolerance [210]. This effect may be attributed to reduced Treg cells and IL-10 production, suggesting proper functioning of CX3CR1⁺ macrophages is essential for local maintenance and survival of tolerance inducing local Treg cells returning from MLNs.

Intestinal macrophages are also involved in maintaining the integrity of the gut mucosa through their high phagocytic activity enabling them to clear bacteria and foreign debris. In addition, macrophages also participate in wound healing through promoting proliferation of epithelial progenitor cells during colonic wound healing. Macrophages establish this role through production of mitogenic factors, metalloproteinase inhibitors and ECM stabilisers [239]. The intestinal epithelium plays an important role in educating these CX3CR1⁺ macrophages to acquire these homeostasis-inducing functions. The mechanisms involved were described in section 1.2.4.3.

Figure 1.2. The intestinal dendritic cell and macrophage compartment. All cDC subsets of the lamina propria arise from the hematopoietic precursor MDP (macrophage-dendritic cell precursor)[24]. In the bonemarrow, MDP differentiates into either the classical DC precursor, or monocytes which can both enter the circulation and infiltrate the gut tissues. The classical DC precursors gives rise to both DC population of the lamina propria and gut associated lymphoid tissue (GALT). The lamina propria is populated by the $CD103^+CD11b^+$ and $CD103^+CD11b^-$ DCs, while the GALT consists of mainly the $CD103^+CD11b^-$ DCs. The $CD103^+CD11b^+$ DCs promote tolerance or protective immunity by upregulating CC-chemokine receptor type 7 (CCR7) after antigen stimulation, which enables transport of antigen to the draining lymph nodes [25, 26]. The monocytes on the other hand, differentiates into $CX3CR1^+$ lamina propria mononuclear phagocytes which are capable of sampling antigen in the lumen through extensions of dendrites passing through epithelial tight junctions. These $CX3CR1^+$ lamina propria mononuclear phagocytes are $CCR7^-$ hence are not capable of migration to the lymph nodes. However they seem to have pro- and anti-inflammatory functions affecting at their site of residence. Figure obtained with permission from [17].

1.2.2 Role of DCs in intestinal inflammation and homeostasis.

In a tolerogenic setting, DCs can induce anergy in antigen-specific T-cells or generate protective FoxP3⁺ regulatory T cells (Treg) in the lymph nodes. This ensures inappropriate inflammatory immune responses to harmless antigens and commensals do not develop. Dysregulation of this fine balance between immunity and tolerance leads to inflammatory disorders such as inflammatory bowel disease (IBD). Two main subtypes of this disease include Crohn's disease (CD), largely arising from a T_H1 response, and ulcerative colitis (UC), largely mediated by IL-5 and IL-13-producing T-cells or natural killer T cells [12]. These two inflammatory diseases relate to an inappropriate and exaggerated immune response to constituents of the gut flora in a genetically predisposed individual. DCs have been shown to play a role in IBD pathogenesis, as suggested by mouse models of DSS-induced colitis [13]. The local microenvironment regulates the function of mucosal DCs through the presence of immune cells, non-immune cells and luminal bacteria [14]. Failure of this microenvironment to condition DCs may promote the development of gut inflammation through: 1) Exuberant priming of colitogenic T-cell responses against commensals and harmless dietary antigens. 2) Sustaining colitogenic T cell reactivity within the inflamed mucosa and lastly 3) By functioning as effector cells releasing pro-inflammatory cytokines [15].

Although CD103⁺ DCs have been shown to promote tolerance at steady-state, they may convert to pro-inflammatory status during inflammation. Pro-inflammatory CD103⁺ DCs have been shown to accumulate in MLNs in experimental model of colitis [229]. These pro-inflammatory DCs down-regulate ALDH1A2 expression and prime inflammatory T_H1-responses, instead of Foxp3 Treg cells [229]. In another study, CD103⁺CD11b⁺ DCs after stimulation with flagellin via TLR5, have been shown to induce pro-inflammatory T_H17 differentiation [230]. In addition, flagellin stimulation also induced IL-23 expression by CD103⁺CD11b⁺ DCs which stimulates IL-22 production by ILC3, needed by the epithelium to produce anti-microbial peptide Reg3γ [231].

In a salmonella infection model, it has been shown that these bacteria recruit CD103⁺ DCs from the deep lamina propria of the small intestines to the epithelium [222], hence CD103⁺ DCs play a role in protecting the gut against microbial insults. Taken

together, CD103⁺ DCs play an important role in establishing tolerance, and at the same time launch protective immune responses during infections.

1.2.3. Other Immune Cells of the Gut Mucosa.

Besides myeloid cells such as DCs and macrophages, a functional gut immune system involves a complex interplay of an underlying network of immune cells. Through priming of these immune cells in the mucosa by the gut microenvironment, they are indispensable in supporting gut immunity. We present below the contributions of these immune cells to gut immunity.

1.2.3.1 Innate lymphoid cells (ILCs)

ILCs are hematopoietic cells belonging to the lymphoid lineage which play important roles in innate immunity and regulation of homeostasis. Unlike T-cells and B-cells, ILCs do not respond in an antigen-specific manner [47], hence ILCs do not resemble adaptive lymphocytes but function as innate components of immunity. In both humans and mice, ILCs reside at barrier surfaces not only in the gut, but also in lungs and skin, where they control tissue homeostasis and inflammation [48, 49]. Their strategic locations within barrier surfaces place them in an ideal position to mount protective innate responses during early infections [303]. ILCs form a phenotypically and functionally heterogeneous population, where they have been classified into 3 groups based on their developmental requirements and cytokine expression [48-51].

Type 1 ILCs

Type 1 innate lymphoid cell (ILC1) are important in innate immunity and regulators of tissue modeling. These cells include classical NK cells and Innate lymphoid cell subset 1 (ILC1) and have been shown to respond to the cytokines IL-12 and IL-15, resulting in the secretion of T_H1 cell-associated cytokines IFN- γ and TNF- α [51]. NK cells have the ability to kill target cells directly through cytotoxic activity mediated by perforin and granzyme B, while ILC1 function is limited to secretion of cytokines in response to stimulus. ILC1 expressed the transcription factor T-bet and can be distinguished from NK cells through the lack of NK markers such as perforin, granzyme B, CD56, CD16 and CD94. ILC1 has been implicated to play a role in pathogenesis of gut mucosal inflammation since elevated numbers were present in inflamed intestines of patients with Crohn's disease [52]. In this study, ILC1 were characterized as NKp44⁺,

c-kit^{low}, T-bet⁺ and responds to IL-12, but not IL-23, by producing enhanced levels of IFN- γ [52]. Another study revealed a distinct ILC1-like subset residing in the intestinal intraepithelial compartment which expresses CD56, CD103, NKp44, NKp46, perforin and granzymeB. These ILC1-like cells expand in numbers during inflammation and respond to the cytokines IL-12, IL-15, but not IL-18 to produce elevated levels of IFN- γ which aggravates anti-CD40-induced colitis in Rag1^{-/-} mice [53]. NKp44, a marker expressed on this intraepithelial ILC1-like subset is also expressed in human ILC3 subset. Despite this similarity, both ILC1 and ILC3 differ significantly based on function, phenotype as well as the requirements of transcription factors for development. For example, it has been described intraepithelial ILC1-like cells requires *NFIL3* and *Tbx21* for development while ILC3s requires *ROR γ t* and *AHR* [51, 53]. In addition, ILC1 express IL-7R α and requires IL-15 for development which both are not required by ILC3s [53]. Depletion of intraepithelial ILC1 ameliorates anti-CD40-induced colitis hence highlighting the role of intraepithelial ILC1 in orchestrating inflammation and disease [53].

Type 2 ILCs

Type 2 innate lymphoid cells (ILC2), are important in early innate response to intestinal helminth infection and have been shown to express IL-7R α , c-kit, CD25, and CD90 [54-56]. These cells respond to IL-25, IL-33 and (thymic stromal lymphopoietin) TSLP, and produce cytokines that are characteristic of TH2-cells such as IL-5 and IL-13 [54-56]. IL-5 and IL-13 produced by ILC2 induces anti-helminth responses required for worm expulsion through increased goblet cell hyperplasia and production of mucus, and increased smooth-muscle contractility [48, 51, 55, 56]. In the lungs, ILC2 promotes airway hyper-responsiveness and tissue-repair in mouse models of influenza virus infection as well as allergy [57-60]. Although the roles of ILC2 in gut allergy and tissue repair has not been extensively studied, the above studies may suggest ILCs have analogous functions in the gut. For instance, in a DSS model of colonic inflammation, ILC2 have been shown to be protective by producing amphiregulin, an epidermal growth factor which supports epithelial repair, and enhancing immunosuppressive functions of Treg cell population hence protecting mice from exacerbated colitis [61]. Lastly besides GATA3 which is highly expressed by

ILC2 [63], the transcription factor ROR α is also required for the development of ILC2 since, ROR $\alpha^{-/-}$ mice lack ILC2 but have normal development of ILC3s [62].

Type 3 ILCs

Type 3 innate lymphoid cells (ILC3) which also includes lymphoid tissue inducer (LTi) cells, requires TSLP, IL-17 and stem-cell factor for development and acquires effector functions in response to IL-23, through secretion of TH17 and TH22 signature cytokines such as IL-17A and IL-22 [65-70]. LTi cells have been well established to organise formation of secondary lymphoid tissues such as Peyer's patches and mesenteric lymph nodes through interactions with stromal cells during embryonic development [71]. In addition it has also been demonstrated, LTi play a role in formation of cryptopatches in intestinal mucosa which transits into mature innate lymphoid follicles (ILFs) in mice after 2 weeks of birth [75]. IL-22 produced by ILC3s is an important cytokine which promotes epithelial repair and regeneration following injury as well as protecting the epithelium from bacterial pathogens through STAT3-induced secretion of anti-microbial peptides (AMP): Reg3 γ , Reg3 β , S100A8, S100A9, lipocalin-2 and calgranulin [76, 77]. This effect of IL-22 limits the translocation of commensals to the mucosa during inflammation [78]. Another cytokine which is involved in the effector functions of ILC3 is IL-1 β . In a mouse model of *Helicobacter hepaticus* infection, IL-1 β has been demonstrated to synergise with IL-23 or IEC derived IL-7 to promote increased production of IL-22 by ILC3s which is important in containing *H. hepaticus* infection through increased production of AMPs by IECs [73-74]. ILC3 development requires expression of the transcription factor retinoid-related orphan receptor- γ t (ROR γ t) since ROR γ t $^{-/-}$ mice were devoid of LTi cells and gut programmed lymphoid tissues such as Peyer's patches and mesenteric lymph nodes [298]. Moreover, ROR γ t $^{-/-}$ mice exhibit increased susceptibility to DSS-induced colitis and formation of compensatory tertiary lymphoid tissues, suggesting an impaired gut immune system [298]. ROR γ t $^{+}$ ILC3 express MHCII and are able to present processed antigen to CD4 $^{+}$ T cells. However, these MHCII $^{+}$ ILC3 lack the expression of costimulatory molecules: CD80, CD86 and CD40, which limits their ability to stimulate CD4 $^{+}$ T cell responses [72]. Moreover, it has also been shown in this study, that presentation of MHCII-loaded antigen by ILC3 to CD4 T-cells, limits rather than stimulate CD4 commensal-specific T-cell responses and consistently depletion of ROR γ t $^{+}$ ILC3 results in spontaneous intestinal inflammation.

ILC3 exhibits a degree of plasticity where they can acquire functional characteristics of ILC1. It has been documented that T-bet⁺CCR6⁺ ILC3 have the ability to upregulate *T-bet* expression followed by acquisition of NKp46 marker. Following this, ROR γ t expression is down-regulated resulting in a conversion of ILC3 into NKp46⁺T-bet⁺ ILC1 which express IFN- γ [78-79]. IL-7, other than being an important cytokine for ILC development, have been shown to stabilize the expression of ROR γ t hence retaining a pool of IL-22-producing ILC3 in the mucosa [79].

Finally, it has been documented that survival of ILC3 in the adult intestinal mucosa diminished significantly only at 3rd week of birth in *Ahr*^{-/-} mice [81]. In this study where intestinal ILC3 has been classified as NK22 (CD3⁻NKp46⁺ROR γ t⁺), LTi4 (CD3⁻NKp46⁻CD4⁺ROR γ t⁺) and LTi0 (CD3⁻NKp46⁻CD4⁻ROR γ t⁺), it is clear that the development and maintenance of intestinal IL-23 responsive ILC3, and their efficacy in producing IL-22, is dependent on Ahr ligands. Moreover, *Ahr*^{-/-} mice succumbed to infection by *C. rodentium* in contrast to WT, suggesting the IL-22 production by ILC3 is important in early defense to *C. rodentium* infection [81].

Figure 1.3. Schematic view of group 1, 2 and 3 ILCs and their related cytokines. The differentiation of ILCs into various groups is dependent on factors high-lighted in rectangular boxes. The stimuli needed for differentiation or production of effector cytokines (rounded boxes) are illustrated on arrows. NCR: natural cytotoxicity triggering receptor (NKp46), TSLP: thymic stromal lymphopoietin, Eomes: eomesodermin. Figure obtained with permission from [64].

1.2.3.2 Tissue resident memory $\alpha\beta$ (TRM) T-cells.

Tissue resident memory T-cells (TRM) reside permanently in peripheral tissues and are phenotypically, transcriptionally and functionally distinct from lymphoid organ and circulating memory-T cell populations [101]. TRMs arise from clonally expanded T-cells which upon leaving the lymph nodes, re-enters the peripheral tissues where they reside permanently with the ability to rapidly respond to cognate antigens. Here, we describe $CD8^+$ and $CD4^+$ TRMs populations in the intestines.

$CD8^+$ TRM $\alpha\beta$ T cells

Priming of naïve T cells in the draining lymph nodes of the gut by antigen-loaded $CD103^+$ DCs during microbial infection by gut pathogens lead to the generation of effector T cells which enters the site of infection from circulation [102]. Entry of these effector T cells into the intestinal mucosa through imprinting of CCR9 expression, promotes clearance of specific cognate pathogens through cytotoxic elimination of infected cells and release of anti-microbial mediators and pro-inflammatory cytokines [103]. However, large numbers of these effector T cells wane off after the course of infection leaving behind a pool of long-lived $CD8^+$ memory T cells with the capability to rapidly respond to re-infections by their cognate pathogens [102]. $CD8^+$ memory T cells have been classified based on their expression of lymph node targeting molecules and function which places them in 2 major subsets: 1) Central memory T cells (TCM) expressing lymph node homing receptors CCR7 and CD62L. 2) Effector memory T cells (TEM), which expresses tissue- homing receptor CCR9 and lacking in CCR7 and CD62L, enabling entry into the sites of infection in the intestinal mucosa [104]. However, in the absence of persisting antigenic stimulation after clearance of pathogens, circulating $CD8^+$ TEM cells gradually lose their expression of intestinal tissue homing molecules and progressively declines in numbers, resulting in a skewing of $CD8^+$ memory T cells towards $CD62L^+$ TCM phenotype [105, 106]. A small population of these infiltrating $CD8^+$ TEM cells have been proposed to consist of $CD8^+$ TEM cells having the capability to infiltrate the intestinal epithelium and differentiate into long-lived $CD8^+$ tissue resident memory (TRM) T cells [107, 108]. Supporting this notion, the prolonged T-cell receptor antigenic stimulation which is readily accessible at the epithelial barrier, although not strictly necessary, promotes $CD8^+$ TEM retention and differentiation into TRM [112-113] through down-regulating responsiveness of $CD8^+$ TEM to CCR7 ligands [109-111]. The retention of

differentiating CD8⁺ TRMs in the epithelium is further supported through local induction of CD103 by TGF- β which is produced abundantly by the enterocytes [114, 115]. CD103, the signature integrin expressed on CD8⁺ TRMs interacts with E-cadherin expressed on epithelium [116]. This interaction is necessary for the survival of CD8⁺ TRMs since CD103 deficient mice have reduced numbers of this T cell population [117]. Besides CD103, CCR9 expression which is sustained on CD103⁺ CD8⁺ TRM cells [105], has also been demonstrated to interact with its chemokine ligand CCL25 which is constitutively expressed by intestinal epithelium. This interaction further promotes the retention of CD103⁺CD8⁺ TRMs in the intestinal epithelium [118].

The strategic position of CD103⁺CD8⁺ TRM T cells lining the epithelial barrier places them at an advantage to quickly respond to subsequent reinfections. In addition, circulating central CD8⁺ and CD4⁺ memory T cells were mostly excluded from the intestinal epithelial barrier in the absence of inflammation hence the intestinal mucosa is not protected against re-infections by pathogens invading from the gastrointestinal route [106]. Hence CD103⁺CD8⁺ TRM T cells forms the first line of defense in protecting the intestinal mucosa from microbial insults through production of effector cytokines such as IFN- γ [119] as well as mounting rapid proliferative recall functions during infections [120].

CD4⁺ TRM $\alpha\beta$ T-cells

While studies are focused mostly on TRM CD8⁺ T cells, CD4⁺T cells have been demonstrated, in both mice and humans, to be the most abundant T cell subset residing in both lymphoid as well as peripheral tissues. In the gut, these CD4⁺ T cells include CD4⁺ memory T cells, where a proportion of it forms a population of permanent resident CD4⁺ TRMs. Besides residing in organized lymphoid tissues such as Peyer's patches, ILFs and GALTs [85-86], CD4⁺ T cells can also be found to populate the intestinal lamina propria as well as in the intraepithelial IEL compartment. However, in the IELs, CD103⁺CD8⁺ T cells, is the major population of IEL T-cells while CD4⁺ T cells constitute to only a minor population [87-89]. Instead, CD4⁺ T cells were found to be the major T cell subset populating the LP where they express CD62L^{lo}CD44^{hi} resembling an effector memory phenotype [90]. It has also been reported, CD69, the TRM marker constitutively expressed on all CD4⁺ and CD8⁺ TRMs [97-100], is

expressed by a vast majority of CD4⁺ T cells in mice small and large intestines [91]. Besides in the intestinal mucosa, CD4⁺ TRMs have also been described in other mucosal tissues such as skin and lungs where they direct protective immune responses through IFN- γ production and recruitment of immune cells to the site of inflammation [82-84].

Intestinal CD4⁺ TRM T-cells, in particular the TH17 subset has been shown to be induced in the LP of mice by a species of segmented commensal known as segmented filamentous bacteria (SFB) [92-93]. TH17 cells are important producers of the cytokines IL-22 and IL-17, where they provide immunity against bacterial pathogens [92-93]. In addition, commensals also mediate the induction of CD4⁺ resident T cells with regulatory functions. Such CD4⁺ T cells include tissue-resident FOXP3⁺ Treg cells, where it has been demonstrated that a vast majority of these were originally conventional T cells converted into a regulatory phenotype upon exposure to commensal bacteria [94]. Infection of the gut with bacterial pathogens such as *Listeria monocytogenes*, likewise have been shown to induce CD4⁺ TRM populations as well as primary T_H1 and T_H2 responses [95].

Central to the orchestration of CD4⁺ and CD8⁺ T cell effector and memory T cell induction, DCs have been shown to mediate this important role [121]. DCs upon acquisition of commensal-derived antigens migrate to mesenteric lymph nodes where they imprint naïve T cells resulting in expansion of effector and memory T cells. These effector and memory T cells then express CCR9 enabling their migration to the lamina propria where they exert their respective functions [96].

1.2.3.3 $\gamma\delta$ T-cells

$\gamma\delta$ T cells represent a minor subset of T cells expressing the TCR which composed of the two glycoproteins γ and δ chains rather than the conventional α and β chains. However, this minor population of $\gamma\delta$ T cells was found in highest abundance in the gut mucosa lining the epithelium hence was characterized as intraepithelial lymphocytes (IEL) [122]. Mouse IELs consist high proportions of CD3⁺ T cells of which ~75% CD8⁺, ~7.5% CD4⁺, ~10% DP (double-positive CD4⁺CD8⁺) and 7.5% DN (double-negative CD4⁻CD8⁻) [123]. TCR $\gamma\delta$ T cells form a substantial population of total IEL CD3⁺CD8⁺ T cells (45%-65%) while the remaining T cells consists of

TCR $\alpha\beta^+$ T cells. Interestingly, while all T cells within the DP and CD4⁺ compartments consist of TCR $\alpha\beta$ T cells, T cells within the DN compartment were solely TCR $\gamma\delta^+$ T cells [123]. In addition, unlike TCR $\alpha\beta$ T cells, IEL TCR $\gamma\delta^+$ T cells do not express the β chain of the heterodimeric CD8 $\alpha\beta$ complex hence CD8 $\alpha\alpha$ homodimer is preferentially expressed on IEL TCR $\gamma\delta^+$ T cells [124].

Cell transfer studies have demonstrated IEL TCR $\gamma\delta^+$ T cells have limited pathogen-specific activity [125, 126]. Nevertheless, the ability of TCR $\alpha\beta^+$ T cells to mediate protection of recipient mice against infection with *Toxoplasma gondii* is dependent on the transfer and presence of CD8 $\alpha\alpha^+$ $\gamma\delta^+$ IEL T cells [126]. In addition, CD8 $\alpha\alpha^+$ $\gamma\delta^+$ IEL T cells have been shown to respond to unprocessed self-antigens which are displayed by stressed or transformed epithelial cells such in the case of viral infections, as well as controlling the activity of $\alpha\beta$ T cells, hence these findings have led to the notion that CD8 $\alpha\alpha^+$ $\gamma\delta^+$ IEL T cells form the first line of defense against invading pathogens [127]. Furthermore, mice lacking in IEL TCR $\gamma\delta^+$ T cells show exaggerated TCR $\alpha\beta^+$ T cell responses resulting in generation of auto-antibodies and destructive $\alpha\beta^+$ T cell mediated pathologies. For example in the case of infection with *Eimeria vermiciformis*, an enterocyte-specific parasite, resulted in containment of parasite in the small intestines. However, mice developed severe bleeding in small intestines due to the uncontrolled TCR $\alpha\beta^+$ T cell responses [133]. This further supports the suggested role of IEL TCR $\gamma\delta^+$ T cells in controlling the effector functions of TCR $\alpha\beta^+$ T cells.

IEL TCR $\gamma\delta^+$ T cells have been shown to produce keratinocyte growth factor (KGF) which is important for epithelial regeneration and repair of epithelial damage mediated by inflammatory immune responses [128]. In addition, TCR δ -chain^{-/-} mice lacking in IEL TCR $\gamma\delta^+$ T cells, showed a reduction in the number of intestinal crypts [129] while introduction of a $\gamma\delta$ TCR transgene improved the mitotic index of intestinal epithelial cells [130]. It was also documented TCR δ -chain^{-/-} mice were more susceptible to 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis. This model employs the use of TNBS which induces severe colonic inflammation when administered intra-rectally in experimental mice. The transfer of IEL TCR $\gamma\delta^+$ T cells to these TCR δ -chain^{-/-} mice protects them from TNBS-induced colitis which correlated with reduced production of pro-inflammatory cytokines such as IFN- γ and TNF- α and increased production of IEL-derived TGF- β [131]. Besides producing

KGF, IEL TCR $\gamma\delta^+$ T cells also express *Tgfb1* and *Tgfb3* which have been shown to be important during recovery of epithelial damage [132]. These findings highlight IEL TCR $\gamma\delta^+$ T cells have an important role in supporting epithelial integrity and repair during epithelial damage.

It has also been reported IEL TCR $\gamma\delta^+$ T cells produce IL-22 alongside ILCs in response to retinoic acid (RA). Administration of RA protects mice from both *Citrobacter rodentium* and DSS induced inflammation through the increased induction of IL-22 [134]. The protective effects of RA were mediated through IL-22-induced upregulation of anti-microbial peptides Reg3 γ and Reg3 β in colonic epithelium. Immunoprecipitation studies to elucidate the mechanism of RA-induction of IL-22 in IEL TCR $\gamma\delta^+$ T cells revealed RA promotes binding of its receptor to the promoter region of IL-22 in the nucleus hence upregulating IL-22 production in TCR $\gamma\delta^+$ T cells [134].

A subset of $\gamma\delta$ T cells have been identified which includes the *Sox13*-dependent V $\gamma 4^+$ $\gamma\delta$ T17 cells which express the scavenger receptor SCART2 [135, 136]. This subset of $\gamma\delta$ T cells have been shown to produce IL-17A and IL-22 [137] where their elevated production in the skin contributes to pathology of chronic skin inflammation known as dermatitis [136]. While limited studies exist in uncovering the effects of IL-17A and IL-22 produced by SCART2 $^+$ V $\gamma 4^+$ $\gamma\delta$ T17 cells in intestinal inflammation, some studies have highlighted that these 2 cytokines protect the gut from colitis [76, 77, 134, 138, 139]. Hence the contribution of SCART2 $^+$ V $\gamma 4^+$ $\gamma\delta$ T17 cells in inflammatory pathologies may be organ-specific.

1.2.3.4 IgA-secreting plasma cells.

The gastrointestinal mucosa is exposed to a myriad of commensals that established itself within the luminal surface shortly after birth [140]. Relatively low bacteria densities; approximately 10^3 to 10^5 per gram of luminal content, can be found populating the proximal section of the small intestine, while higher bacterial densities can be found populating the colons at 10^{10} to 10^{12} organisms per gram, which consists of >1000 species of commensals [141]. Containment of these commensals without the ill-effects of bystander tissue damage, while preserving their mutualistic relationship with the host is important hence the gastrointestinal immune system has evolved

specific mechanisms to establish this feat. A key mechanism to contain these commensals without the need to trigger inflammation is through the production of immunoglobulin A (IgA) [142]. IgA dimers produced by B cells in the lamina propria is transported from the basolateral side of epithelial cells through binding to polymeric Ig receptor (pIgR) and then transcytosed to its apical side towards the lumen, hence generating secretory IgA (SIgA) which has several protective roles [143]. SIgA secreted into the mucus protects the epithelial surface through neutralization of commensal-derived proinflammatory epitopes and dietary antigens which results in peaceful co-existence of bacterial communities within specific intestinal sections [144].

Antigen-specific IgA responses are induced predominantly in the Peyer's patches where interaction of Ag-specific B and T cells occur within germinal centers. B-cells within these germinal centers express Activation-Induced Deaminase (AID) which is essential for generating a huge repertoire of Ig specificity through somatic hyper-mutations [156]. TGF- β which is highly expressed in Peyer's patches [157], together with CD40L expression on DC-activated CD4⁺ T cells stimulate class-switch recombination events in cognate B cells leading to generation of Ag-specific IgA⁺ B cells [158-159]. Mice lacking in Peyer's patches and MLN but retained innate lymphoid follicles (ILFs) were unable to mount antigen-specific mucosal IgA immune responses after oral immunization, although having unaltered IgA production in the intestines [145]. Conversely, mice treated post-natally with LT α depleting antibodies (anti-LT β R-Ig), resulting in depletion of ILFs but not Peyer's patches or MLNs, were able to mount antigen-specific mucosal IgA immune responses [145]. However, when mice were treated during gestation with anti-LT β R-Ig resulting in depletion of only Peyer's patches but not MLN or ILFs, retained IgA-producing plasma cells in the lamina propria [145]. Hence this highlights the non-absolute requirement of Peyer's patches in inducing specific IgA immune responses since this induction may probably occur in the MLNs. Interestingly, one study have shown that IgA production can be mediated by mechanisms that are entirely independent of Peyer's patches, MLNs and ILFs [146]. In this study, ROR γ t deficient mice which lacked LT α cells needed for the formation of intestinal follicular lymphoid structures were tested for production of gut mucosal IgA. Remarkably, these mice lacking in Peyer's patches, MLNs and ILFs retained a population of IgA-producing plasma cells in the lamina propria. Hence this result shows that the lamina propria can also serve as an inductive site for IgA-

producing plasma cells. Indeed, it was demonstrated the lamina propria consists of naïve IgM⁺ B cells which form precursors to IgA-producing plasma cells, originating from the bone marrow [147, 148]. Another IgM⁺ B cell population was identified as the gut-experienced IgM⁺ B cells migrating from Peyer's patches to the lamina propria [148]. The detection of Activation-Induced Deaminase (AID) mRNA transcript in both IgM⁺ and IgA⁺ B-cells in the lamina propria suggests that lamina propria resident IgM⁺ B-cells may undergo class-switching to IgA-producing plasma cell *in situ* [149]. This notion is further supported by the observation that AID^{-/-} mice lack IgA producing plasma cells in the lamina propria [150].

The activation of IgA-producing plasma cells in the lamina propria may not involve T cells. For instance, lamina propria DCs may present antigens directly to B-cells resulting in activation through interactions with B cell receptors and TLRs and also through DC expression of B-cell activating factors (BAFF) and a proliferation inducing ligand (APRIL), which are structurally and functionally related to CD40L [151-153]. It has been demonstrated in both mice and humans, BAFF and APRIL stimulates IgA heavy-chain class-switch recombination in B-cells independently of CD40L [154, 155] hence do not require interactions with T cells.

IgA secreted by plasma cells in the intestinal mucosa serves a wide range of protective functions. IgA generated through CD4⁺ T cells generally are thought to contain infection of pathogens on intestinal mucosal surfaces [160, 161]. Conversely, IgA generated through T cell-independent mechanisms are thought to confine commensals via “immune exclusion” [160]. However, this definition is not absolute since in a rotavirus infection, T cell independent IgA production has been shown to mediate some degree of protection [162]. Conversely, IgA responses generated through T cell dependent mechanism, have been shown to control harmless commensal bacteria [163]. Taken together, IgA production by intestinal plasma cells is an important mechanism in maintenance of effective epithelial barrier in both steady state and inflammation.

1.2.4 The role of IECs in barrier function and regulation of immune homeostasis.

The intestinal epithelium serves as an effective barrier which separates the intestinal mucosa from the external environment consisting a myriad of commensals as well as dietary antigens which occupy the luminal space. Specialized lineages of epithelial cells span the entire surface area of the gastrointestinal mucosa to maintain this segregation, which is key to establishment of peaceful coexistence between host and commensals. Intestinal epithelial cells (IECs) play an active role in performing this feat through sensing of microbe-derived stimulus and delivering the appropriate barrier responses resulting in tolerance or protective inflammatory immune responses. This highlights the fundamental importance of IECs in intestinal immune homeostasis. The complex yet interesting mechanisms of how IECs maintain and regulate epithelial barrier functions, hence preventing development of uncontrolled intestinal inflammation that would otherwise result in IBDs, will be highlighted in this section.

1.2.4.1 IEC secretory defences

The IEC consists of a majority of absorptive enterocytes which are specialized in metabolic and digestive functions. Other IEC lineages exist within the IEC compartment which enables the epithelium to perform a diversity of functions. For instance, secretory IECs which includes Paneth cells and Goblet cells secrete anti-microbial peptides (AMPs) and mucins respectively towards the lumen which forms a physical and biochemical barrier against effacing commensals hence effectively preventing their contact with the epithelium and reaction with underlying immune cells [164, 165] . This protects the mucosa from infection and limiting prolonged exposure of the underlying immune cells from potentially inflammatory stimuli.

Mucin 2 (MUC2), the most abundant form of mucin in the gut, is a highly glycosylated protein secreted by goblet cells, forms a large component the mucus layer overlying the epithelium surface [166]. Mice deficient of MUC2 developed spontaneous colitis and increased susceptibility to inflammation-induced colorectal cancer [167, 168], thus highlighting the importance of the mucus barrier in intestinal homeostasis. Besides MUC2, goblet cells also produce trefoil factor 3 (TFF3) and resistin-like molecule- β (RELMB). TFF3 stabilizes the integrity of the mucus layer through crosslinking of MUC2, and also stimulates repair and migration of IECs rendering them resistant to apoptosis [169, 170]. RELMB on the other hand, stimulates

the production of MUC2, and regulates innate cell responses such as macrophages; and also adaptive T cell responses during inflammation [171, 172].

The absorptive epithelium itself, other than fulfilling nutrient absorptive functions, is capable of producing anti-microbial peptides which further re-inforces the biochemical barrier. These anti-microbial peptides include the C-type lectin regenerating islet-derived protein 3 γ and β (REG3 γ and REG3 β) [307-309]. REG3 γ targets exclusively Gram⁺ bacteria through surface recognition of exposed carbohydrates moieties on bacterial peptidoglycan and has been demonstrated to mediate effective anti-bacterial properties against *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* by disrupting their cell-surfaces [173, 174]. The exact role of Reg3 β in bacterial infections remains unclear. However, it has been demonstrated that Reg3 $\beta^{-/-}$ mice failed to contain Gram⁻ Salmonella enteritis infections while no difference in terms of outcomes were observed during infections with Gram⁺ *Listeria monocytogenes* as compared to WT. This shows that Reg3 β may mediate protective roles against infections by Gram⁻ bacteria [175]. The epithelium have been shown to also produce two S100 family calcium-binding proteins S100A8 and S100A9 which exists mainly as a S100A8/S100A9 heterodimer [176, 177]. The anti-microbial activities of these AMPs is mediated through inhibition of bacterial adhesion to the epithelium by zinc chelation [178-181]. Paneth cells by contrast, other than their ability to secrete REG3 γ and REG3 β , also secrete other AMPs such as defensins, cathelicidins and lysozymes in the intestinal crypts [182-183]. These AMPs such as defensins and cathelicidins mediate their anti-microbial functions by disrupting the membranes of bacteria through formation of pore complexes [182, 184]. Entrapment of these AMPs within the mucin scaffold forms a layer of concentrated anti-microbial activity directed at the epithelial surface, which bolsters the physical barrier. Hence effectively maintaining the segregation between commensals and the host immune system.

Finally, IECs have the ability to transport secreted IgA (SIgA) directly across the epithelial barrier through polymeric immunoglobulin receptor (pIgR)-dependent transcytosis. IgA dimers produced by B cells in the lamina propria is transported from the basolateral side of epithelial cells through binding to polymeric Ig receptor (pIgR) and then transcytosed to its apical side towards the lumen. SIgA secreted into the mucus layer protects the epithelial surface through neutralization of commensal-

derived proinflammatory epitopes and dietary antigens which results in peaceful co-existence between bacterial communities and host [144].

Figure 1.4. The functional intestinal epithelial barrier. Secretion of AMPs by absorptive epithelium and Paneth cells, as well as mucins by Goblet cells, reinforce the physical barrier which is important in segregation between commensals and host. The transport of SIgA to the luminal space further supports this barrier function. The transport of bacteria across this barrier involves specialized lineage of epithelial cells known as M-cell, which are present on the epithelium of Peyer's patches and lymphoid follicles. Transcytosis of live bacteria across the barrier results in subsequent presentation to dendritic cells. Figure obtained with permission from [186].

1.2.4.2 IECs as sentinels in intestinal homeostasis.

Other than contributing to epithelial barrier function, IECs regulate intestinal homeostasis through their ability to sense as well as integrate commensal-derived antigens and directing appropriate anti-microbial or tolerance-inducing responses. The recognition of commensal antigens by IECs is possible through the expression of pattern recognition receptors (PRRs). These PRRs include 1) Toll-like receptors (TLRs) [187] 2) RIG-1-like receptor (RLR) [188, 189] and 3) NOD-like receptors (NLR) [190, 191] which enables IECs to sense a wide array of microbial ligands or endogenous signals present during pathogenesis. Due to the abundance of commensals

in the gut, it is important for IECs to maintain a state of selective responsiveness to these commensals so as to establish immune tolerance.

Recognition of microbial products through TLR signaling in IECs, have been shown to promote epithelial tissue homeostasis and repair after damage induced by DSS [192]. This study also concluded that mice depleted of commensals or lacking in specific components of TLR signaling were more susceptible to DSS-induced colitis. In addition, TLR signaling in IECs induced during recognition of microbes, leads to a host of IEC protective responses such as upregulation of heat-shock proteins, EGF receptor ligands [192] and trefoil peptide 3 secretion [193]. These studies highlight the importance of TLR signaling in intestinal homeostasis and repair.

Other categories of TLRs which enables the sensing of microbial signals and regulating IEC responses include NLRs and RLRs. These TLRs have also been shown to regulate intestinal homeostasis [194-196]. NLRs recognize bacteria-derived muramyl dipeptides through its nucleotide-binding oligomerisation domain 2 (NOD2) and its identification in IECs has been associated as a susceptibility locus for Crohn's disease [197]. The different forms of NLRs expressed by both immune cells and epithelium, such as the pyrin domain-containing: NLRP3, NLRP6 and NLRP12 as well as the CARD-containing: NLRC4 associates with caspase 1 to form inflammasomes which regulates intestinal inflammation and epithelial repair in knock-out mice models [194, 195, 198]. In particular, it has been demonstrated through recognition of bacterial flagellin by NLRC4 expressed by CD8⁺ DCs, induces the formation of inflammasomes leading to secretion of active IL-18 [381]. This cytokine stimulates protective effectors functions of IEL CD8⁺ resident memory T cells in a non-cognate manner through secretion of IFN- γ [382-383]. In addition, IL-18 has also been shown augment the secretion of a pro-inflammatory cytokine such as TNF- α by macrophages [313], hence demonstrating the multifaceted roles of this NLR-induced cytokine.

1.2.4.3 Regulation of immune cells by IECs.

Other than the ability to sense and integrate microbial signals from the external environment, IECs also produce immunoregulatory mediators important for maintenance of tolerance against harmless commensals and dietary antigens. These

immunoregulatory mediators tolerize immune cells in the mucosa leading to non-responsiveness to these harmless antigens during non-inflammatory conditions. The commensals have been shown to shape tolerance inducing functions of IECs. For example, IEC production of thymic stromal lymphopoietin (TSLP) [32], transforming growth factor- β (TGF β) [199], a proliferation inducing ligand (APRIL) and B cell-activating factor (BAFF) [200, 201] were known to be stimulated by commensals via PRR recognition. Here, we describe the ‘conditioning’ of various immune cells in the gut mucosa by IECs.

CD103⁺ DCs and macrophages.

The ability of intestinal CD103⁺CD11c^{hi} DCs to upregulate homing receptors on lymphocytes, to drive peripheral generation of FOXP3⁺ T_{Reg} cells and to support class-switching to IgA, resulting in an establishment of an overall immune non-responsiveness towards commensals and harmless dietary antigens, is shaped by the intestinal epithelium. Communication between the intestinal epithelium and DCs is likely to be integral in this conditioning [31]. Activation of nuclear factor- κ B (NF- κ B) expression on intestinal epithelial cells, as a result of commensal microflora signaling through pattern-recognition receptors, enhances their production of TSLP [32]. TSLP and other epithelial-cell derived factors can act on DCs to down regulate IL-12/23p40 production in response to bacterial stimulation [33], and preferentially drive non-inflammatory T helper 2 (T_H2)-type responses [32]. IL-10 and TGF β derived from multiple sources may also have a role in limiting the responsiveness of intestinal DCs to bacterial or other activation signals. Retinoic acid derived from epithelial metabolism of dietary retinol by the enzyme aldehyde dehydrogenase family 2 (ALDH1A2) enhances the effect of TGF β -mediated induction of FOXP3 in T cells [202-204]. Defective conditioning of DCs in the intestine may contribute to the pathogenesis of inflammatory bowel disease.

Figure 1.5. Conditioning of DCs in the intestine involves interaction of DCs with the microenvironment and factors derived from the intestinal epithelium. Figure obtained with permission from [34].

Tolerized CD103⁺CD11c^{hi} DCs as a result of conditioning by the epithelium, migrates to mesenteric lymph nodes after acquisition of antigens in the lamina propria, for presentation to naïve T-cells and B-cells [205, 206]. It has been shown these migrants from the lamina propria deliver commensal antigens and apoptotic IECs to the mesenteric lymph nodes during non-inflammatory conditions [27]. Migration of these DCs to the lymph nodes is dependant on CCR7 [20]. Presentation of antigens by these migratory DCs in the absence of inflammatory signals to T and B cells result in tolerance hence maintaining a non-inflammatory environment in the gut. Lamina propria, Peyer's patches and mesenteric lymph node DCs have been shown to have a higher propensity to induce FOXP3⁺ T cells *in vitro* when compared to splenic DCs in

the presence of IEC-produced TGF β [28]. Gut homing potential of CD4⁺ and CD8⁺ T cells activated in the mesenteric lymph nodes were imprinted by CD103⁺ DCs through promoting the expression of $\alpha_4\beta_7$ -integrin and CCR9 on T cells [29]. Finally, Peyer's patch DCs have a role in class-switching to non-inflammatory IgA which lines mucosal surfaces [30]. Mediators produced by intestinal DCs implicated in IgA class-switching includes IL-6, TGF β , IL-10 and APRIL (proliferation inducing ligand) [30].

Unlike CD103⁺CD11c^{hi} DCs, intestinal CD11c^{low}F4/80⁺CX3CR1^{hi} resident macrophages do not express CCR7 hence were not able to migrate to mesenteric lymph nodes during steady-state but were retained in proximity to IECs. These macrophages behave as sentinels, phagocytosing bacteria which has breached the epithelial barrier [205, 207]. Through TLR recognition of commensals, IECs condition CD11c^{low}F4/80⁺CX3CR1^{hi} resident macrophages to extend transepithelial dendrites towards the lumen hence enabling the sampling of exogenous antigens in the luminal space [208, 209]. These resident macrophages have been shown to promote mucosal tolerance through the maintenance and expansion of Treg cells [210]. IL-10 produced by these macrophages is a potent cytokine for suppressing colitogenic T cells, as well as promoting immune-suppressive Treg cell functions [211, 212]. The maintenance of tolerogenic properties of these CD11c^{low}F4/80⁺CX3CR1^{hi} resident macrophages were dependent on IEC production of secreted RA, TGF β and TSLP [32, 199, 213], and contact-dependent interaction with an IEC-expressed integrin ligand: semaphorin 7A which stimulates CD11c^{low}F4/80⁺CX3CR1^{hi} resident macrophages to secrete IL-10 [214]. Hence IEC play an important role by acting as an initial sensor which 'instructs' the next course of action to DCs and macrophages, thereby promoting intestinal immune homeostasis.

ILCs

Studies describing the conditioning of ILC1 and ILC2 by IECs were limited. However, ILC1 residing in intestinal epithelial compartment, responds to IL-12 and IL-15 derived from IEC-conditioned CD103⁺ DCs, by producing IFN- γ and TNF α [51].

IECs support the proliferation and activation of ILC2 through the cytokines IL-25, IL-33 and TSLP [54-56, 215] and have been shown to produce IL-5 and IL-13 which are important in early innate response against gut helminth infections [48, 51, 54-56].

ILCs3 are indirectly regulated by IECs which through sensing of commensal-derived antigens produce IL-25 leading to suppression of IL-23 by DCs and macrophages resulting in decreased IL-23-dependent ILC3 production of IL-22. However, in an inflammatory setting where the epithelial barrier is damaged resulting in increased translocation of commensals to the mucosa, IECs downregulate IL-25 production and through stimulation of macrophages and DCs with commensal-derived ligands, results in increased levels of IL-23. ILC3s then respond to the increased IL-23 to produce IL-22 which restores epithelial barrier function through repair of IEC layer and stimulation of AMP production by IECs to contain commensals to the lumen [72]. Through sensing of commensals via PRRs, IECs produce the cytokine IL-7 which stabilizes the expression of ROR γ t on ILC3 hence supporting IL-22 secretion by ILC3 [80, 216].

Tissue-resident T cells.

Tissue-resident T cells of the gastro-intestinal mucosa residing within the epithelial layer (also known as IELs) includes CD4⁺ and CD8⁺ conventional T cells, $\gamma\delta$ T cells and NKT cells. The contribution of the environment where these immune cells reside, on the regulation and functions of these tissue-resident T cells, have not been widely studied. However, the adhesion of CD8⁺ tissue-resident memory (TRM) T cells to the epithelium via interaction between CD103 on T cells and E-cadherin on IECs promotes the retention of these TRMs to the intestinal epithelium [116]. In addition, mouse IECs have been described to select the survival of high affinity CD8 $\alpha\alpha$ ⁺ T cell populations in IELs through interaction with IEC-expressed thymus leukemia antigen (TLA), a MHC class 1-like molecule [217]. This selection leads to the retention of high-affinity CD8⁺ TRM cell pool, allowing efficient memory responses to secondary infections [217].

1.3 Inflammatory bowel diseases – breakdown of intestinal immune homeostatic regulation.

Crohn's disease (CD) and ulcerative colitis (UC) can be categorized as Inflammatory bowel diseases (IBDs) affecting the gastrointestinal tract. Ulcerative colitis solely affects the mucosal layer of the colons, spreading upwards from the rectum to the proximal colon in a diffused pattern. However, in Crohn's disease, any part of the gastrointestinal tract can be affected, while the ileum being the most frequently affected region. Inflammation in Crohn's disease is transmural and patchy in contrast to ulcerative colitis, and exhibits a cobblestone-like appearance. The precise factors leading to these IBDs remain unclear, but it has been shown that the pathogenesis of IBDs is complex and involves multiple factors that can be grouped into 3 categories: genetic factors, the host immune system itself and the environment dominated by commensal bacteria [240-241]. In this section, we illustrate the known mechanisms supporting pathogenesis of this multifactorial inflammatory disease.

1.3.1 Defective secretory functions of IECs.

Section 1.2.4.1, illustrates the secretory functions of IECs which is important in bolstering the innate epithelial barrier function. In brief, IECs consist of multiple specialized lineages of epithelial cells, each performing differing roles such as nutrient absorption and secretion of mucins, anti-microbial peptides (AMPs). The secretion of mucins by goblet cells coat the luminal surface of IECs with a thick mucus-layer that is highly impermeable to commensals. The mucus layer also allows the retention of AMPs secreted by Paneth cells and absorptive enterocytes, and SIgA to the mucus scaffold thereby further reinforcing the biochemical barrier. The importance of this mucin secretion by goblet cells has been highlighted in studies using mice with decreased numbers of goblets cells and defective MUC2 production, resulted in spontaneous development of colitis [31, 242]. In addition, loss-of-function mutations in the transcription factor TCF4, important for maturation of Paneth cells, have been associated with ileal Crohn's disease [243]. Besides secreting mucins and AMPs, IECs also shape CD103⁺ DC tolerogenic functions resulting in a state of hyporesponsiveness towards commensal and dietary antigens and the mechanisms how IEC accomplish this is described in section 1.2.4.3.

1.3.2 Aberrant pattern recognition receptors (PRR) signaling.

The sensing of commensals via PRR expressed on IECs and immune cells such as DCs and macrophages, maintains basal tonic PRR activation which drives proper functioning of IEC barrier [187, 244, 245]. Aberrant PRR signaling may contribute to pathogenesis of IBDs. This notion is supported by studies in mice lacking TLR2, 4, 5, 9 and MyD88 showed increased susceptibility to DSS-induced colitis as exhibited by defective tissue repair and clinical signs of disease including increased body-weight loss and histological scores of colitis [187, 245]. In addition, the sensing of commensals by Paneth cells through TLRs, have been shown to be important in stimulating the production of AMPs such as defensins and REG3 γ in Paneth cells resulting in containment of commensals to the luminal space [187].

Another class of PRRs important in maintaining barrier function includes the cytosolic NOD-like receptors (NLRs). Mice lacking in *Nod1* or *Nod2* genes show defective production of defensins and increased susceptibility to exacerbated colitis [246, 247]. In addition, NLR-dependent inflammasome activation contributes to repair of epithelial damage and resistance to colitis since mice lacking in NLRP3 or other components of the inflammasome showed qualities of exacerbated colitis [248-250]. The activation of inflammasomes result in activation of caspase-1 which regulates two pro-inflammatory cytokines IL-1 β and IL-18 [251]. Since exogenous administration of IL-18 attenuated DSS colitis in caspase-1 and IL-18 deficient mice, this highlights the protective role of IL-18 in protecting epithelial damage by DSS [252].

While tonic PRR stimulation in steady-state is necessary for protective epithelial barrier responses, continuous and prolonged PRR stimulation drives chronic intestinal inflammation. This is supported by a study demonstrating the selective knock out in hematopoietic cell expression of MyD88, resulted in less severity of intestinal inflammation when mice were infected with *Helicobacter hepaticus* [254]. However, within the hematopoietic cell compartment, MyD88 signaling in T cells were not required for expression of pathogenic effector molecules [254], whereas MyD88 signaling in dendritic cells is required for T cell proliferation, driving chronic intestinal inflammation [255].

1.3.3 Pathological effector molecules of inflammation

TNF α and IL-6

The pro-inflammatory molecules TNF- α and IL-6 secreted by myeloid cells in the gut have been shown to contribute to the pathogenesis of IBDs. This is supported by the studies that depletion of TNF- α and IL-6 using depleting antibodies ameliorated the pathology of chronic intestinal inflammation [240, 253].

IL-23 (p19/p40), T_H1 and T_H17 responses

The dysregulation in IL-12 p40 and T_H1 responses were found in earlier studies to be associated with Crohn's disease [240]. Consistently, administration of neutralizing antibodies to T cell cytokines such as IL-12p40 and IFN- γ attenuated colitis in mice hence highlighting the roles of these cytokines to inflammation [240]. However, IL-23p19 also dimerises with the IL-12p40 sub-unit to form a IL-23 heterodimer. IL-23 has been shown to be a key cytokine in mediating chronic intestinal pathology [256] by promoting damaging T_H1 and T_H17 cell responses and excessive innate lymphoid cells activation [256, 257]. This is consistent with the observation that the IL-23-induced T_H1 and T_H17 cytokines such as IL-17, IFN- γ and IL-22 were upregulated in chronically inflamed intestines [256, 257]. In a CD40-induced model of colitis, deficiency in IL-12p35 but not IL-23p19 in a Rag1^{-/-} background resulted in colitis marked by increased colonic expression of TNF- α , MCP and IL-6 [36]. This result highlights IL-23 other than promoting colitis-inducing T_H1 and T_H17 responses [256], also drives intestinal inflammation independently of T-cells.

T_H17 cells, along with their signature cytokines were elevated in the inflamed tissues of the intestine, suggesting their role in IBDs [256, 257]. These T_H17 cytokines include IL-17A, IL-17F, IL-21 and IL-22 [256]. The role of IL-17A and IL-17F have been shown to mediate inflammation and upregulation of cytokines and chemokines that promote neutrophil recruitment [256]. However, the role of IL-17A and IL-17F in mice subjected to acute DSS inflammation seems to bear conflicting results. It has been observed IL-17A protects mice from acute DSS inflammation, whereas IL-17F play a pro-inflammatory role [258]. In contrast, a colitis model using *H. hepaticus* infection suggests IL-17A exerts pro-inflammatory functions leading to innate immune colitis in mice [65]. Furthermore, in studies using T-cell-transfer models of colitis, IL-

IL-17A and IL-17F do not play a role in pathogenesis of colitis [259]. Hence IL-17A and IL-17F bears multifaceted functions which is dependent on the context of inflammation.

NLRs and inflammasomes-associated cytokines.

Recently, association studies of genes relating to IBD susceptibility have pointed out that polymorphisms in *NLRP3* and *IL18RAP* gave high incidences of developing autoinflammatory diseases [260]. Hyperactivation of NLRP3 leading to increased mucosal production of its associated cytokines IL-1 β and IL-18 have been shown to manifest in patients with IBDs [240]. Consistently, IL-18^{-/-} mice were resistant to TNBS-induced colitis. This highlights the capacity of IL-1 β and IL-18 to aggravate chronic intestinal pathology. In addition, IL-1 β and IL-18 have also been shown to promote effector functions of proinflammatory T_H17 and T_H1 responses respectively [252, 261]. Hence NLRs can contribute to pathology of IBDs through their associated cytokines IL-1 β and IL-18.

Immune-suppressive TGF- β and IL-10

TGF- β is highly expressed in the intestinal mucosa and has been shown to be important in modulating inflammatory immune responses, hence promoting a state of tolerance to the myriad of commensals and food antigens existing in the lumen [262]. Mice lacking in *Tgfb* developed lethal inflammatory disease with similar to Foxp3^{-/-} mice [262]. While the intestinal IECs production of TGF β is essential for maintaining intestinal tolerance, T cells also contribute to production of this cytokine. Mice harbouring T-cells deficient in either TGF β or its receptor showed inability to maintain immune homeostasis [262]. In addition, T cells that are non-responsive to Treg-derived TGF β , escapes this ‘conditioning’ and becomes inflammatory effector T cells. This observation is consistent with the observation that T cells from IBD-affected intestines are refractory to TGF β effects, through the TGF β negative regulator SMAD7 [263].

IL-10 is another anti-inflammatory cytokine of the gut which is widely produced by a wide range of hematopoietic cells which includes myeloid cells, T cells and B cells. Deficiency in this cytokine results in colitis pathogenesis in mice [264]. T cell populations which produce IL-10 includes FOXP3⁺ and FOXP3⁻ CD4⁺ T cells. Of note

the colon harbours a majority FOXP3⁺IL-10⁺ T cell population while the small intestine contains both FOXP3⁺ and FOXP3⁻ IL-10⁺ T cells [264]. Intestinal commensals, more specifically *Clostridium spp* have been shown to induce IL-10 production by T cells [265]. Other than T cells, myeloid cells such as macrophages also contribute to IL-10 production. IL-10 produced by macrophages supports FOXP3 T reg cells in suppressing colitis [266]. Mutations in IL-10 receptor genes: IL10RA and IL10RB resulted in early onset of IBD [267]. Taken together, these finding highlight the homeostatic role of IL-10 and commensals are important in its induction.

1.4 Studying the Role of DCs in Intestinal Inflammatory Disorders.

Crohn's disease and ulcerative colitis can be categorized as Inflammatory bowel diseases (IBDs) affecting the gastrointestinal tract. Ulcerative colitis affects the sole mucosal layer of the colons, and spreads upwards from the rectum to the proximal colon in a diffused pattern. In Crohn's disease, any part of the gastrointestinal tract can be affected, while the ileum being the most frequently affected region. Inflammation in Crohn's disease is transmural and patchy in contrast to ulcerative colitis exhibiting a more diffused pattern.

These aberrant mucosal immune responses in the gastrointestinal tract points to failure in regulating homeostasis of gut inflammatory environment. DCs have been suggested in several human and murine models to play a pathogenic role in development of IBDs. As mentioned in section 1.2.4.3, the function of mucosal CD103⁺ DC is conditioned by factors in the local microenvironment, hence dysregulation in any of these factors can lead to abnormal DC functioning resulting in IBDs.

1.4.1 Experimental Model of Colitis.

Several studies using mouse models of intestinal inflammation resembling IBD have shed light on a possible pathogenic role of DCs in establishment or maintenance of colitis. For instance, DC activation via the costimulatory molecule CD40 causes gut inflammation in the absence of B and T cells via the release of inflammatory cytokines, including IL-23 and IL-17 [36]. For our study, we used the DSS-induced acute colitis model to study the role of our CD103⁺ CD11b⁻ and CD103⁺CD11b⁺ DC subset in intestinal inflammation.

1.4.2 Murine DSS-Induced Colitis Model of Innate Intestinal Inflammation

Dextran sulfate sodium (DSS), a negatively charged polymer of glucose with engrafted sulfate groups, induces colitis in mice. DSS associates with medium-chain-length fatty acids (MCFAs) such as dodecanoate, which is enriched principally in the distal colonic lumen. DSS complexed to MCFAs form nanometer-sized vesicles of approximately 200nm in diameter that fuse with colonocyte membranes. In the cytoplasm, these vesicles affect major epithelial cell pathways and consequently lead

to disruption of intestinal barrier [40]. The disruption of epithelial barrier results in breach of commensal constituents into the lamina propria, activating underlying immune cell network leading to intestinal inflammation. A graphical representation as follows:

Figure 1.6. Mechanism of DSS-induced colitis in mice. Disruption of colonic epithelium by DSS leads to infiltration of commensals from the intestinal lumen into the lamina propria where they interact with underlying immune cell network leading to inflammatory response. Figure obtained with permission from [41].

The administration of DSS dissolved in drinking water to mice, for several days induces a colitis that is characterized by hematochezia (blood in the stool), body weight loss, shortening of the colons, mucosal ulceration, and infiltration with neutrophil granulocytes [37]. Histopathological analysis reveals extensive crypt and epithelial cell damage with ulceration, tissue edema, hyperplasia (swelling) of lamina propria and activation of the innate immune system. Mucosal damage is observed in both wild-type and severely combined immunodeficient (SCID) mice during the acute phase of colitis, indicating that adaptive immune response might not be involved in the induction of acute inflammation [38]. Thus, acute DSS-induced acute colitis model is often used for the study of the innate immune system in colitis.

1.4.3 The Diphtheria Toxin Receptor-Diphtheria Toxin (DTR-DT) System.

The diphtheria toxin (DT) produced by *Corynebacterium diphtheriae* is a potent toxin that, once internalized into a mammalian cell, efficiently blocks protein synthesis causing rapid cell death (Figure 1.7a). The DT polypeptide consists of two subunits: the B subunit is responsible for binding to the DT receptor (DTR), a membrane-anchored form of the heparin-binding epidermal growth factor (hb-EGF); binding of the B subunit to the DTR facilitates the entry of the A subunit into the cell, where it inactivates the elongation factor 2 via ADP ribosylation, preventing protein synthesis

and causing cell death via apoptosis [42]. Owing to three amino changes in the extracellular region of the DTR that is crucial for binding of the B subunit of the toxin, rodent cells are at least 10^3 - 10^5 times more resistant to DT than human or other primate cells. Thus, engineered expression of the high-affinity human or simian receptor by a particular rodent cell type can be used as a powerful tool to deplete that population in vivo (Figure 1.7b). The use of this DTR-DT system is especially attractive to immunologists because DT induces apoptosis in target cells, including terminally differentiated cells such as DCs. Clearance of apoptotic cells by low doses of DT does not induce inflammation, hence the depletion can be considered to occur in an immunological steady state condition.

Figure 1.7a. Mechanism of the DTR-DT system. DT efficiently blocks protein synthesis causing rapid cell death. Figure obtained with permission from [43].

Figure 1.7b. DTR-DT system of specific ablation of cell-type in vivo. Figure obtained with permission from [43].

More recently, the availability of mice expressing the diphtheria toxin receptor under the control of the CD11c promoter has allowed the selective depletion of DCs to enable study of DC role in the development of DSS-induced colitis [39]. CD11c, also known as Integrin, alpha X (ITGAX) is a type I transmembrane protein found at high levels on murine DCs, monocytes, macrophages, neutrophils, and some B cells. In this mouse model, administration of DT leads to depletion of most DC subsets in the intestine. According to this study, CD11c⁺ DC ablation prior to DSS treatment was shown to ameliorate DSS-induced colitis. In addition, consistent with this observation, transfer of bone marrow derived DCs prior to DSS-treatment was shown to exacerbate DSS-induced colitis. This suggests that DCs have a pro-inflammatory role in intestinal inflammation. However, if TLR9 ligands (ISS-oligodeoxynucleotide) were administered to mice prior to colitis induction, mice were protected from DSS-induced colitis. Consistently, DC ablation in these mice during DSS-induced colitis exacerbated the disease [44]. This leads to the authors proposing that in the control mice, activation of DCs with TLR9 ligands led to production of IFN β , which in turns inhibited the production of pro-inflammatory cytokines by macrophages. These findings suggest that depending on their mode of activation, cDCs can enhance or attenuate the severity of DSS-induced colitis independently of T cells.

However in these studies, the administration of DT in CD11c-DTR mice results in ablation of the general CD11c^{hi} DC population rather than a specific subset. In addition, it has been recently found that CD11c expression is not restricted to conventional DCs, as it is also expressed in plasmacytoid DCs (pDCs), NK cells, activated CD8⁺ T cells, monocyte-derived inflammatory DCs, as well as a subset of F480^{hi} macrophages, so it raises the question whether the outcomes observed in these studies are a result of depletion of cell types other than cDCs or indeed by the cDCs themselves. Furthermore, cDCs form a heterogeneous population whose functions may be subset-specific. Therefore the generation of new DTR mice strains capable of depleting specific intestinal DC subsets for studying their role in DSS-induced colitis remains to be carried out.

1.4.4 Generation of Clec9A-DTR and Clec4a4-DTR Mice.

Clec9A-DTR

To study the function of specific subsets of intestinal DCs in our DSS-induced colitis model, mice capable of depleting specific DC subsets have been generated in our lab. In particular, the transgenic Clec9A-DTR mice has been developed to ablate CD8⁺ lymphoid DCs as well as the peripheral tissue equivalent CD103⁺ CD11b⁻ DCs. Generation of these Clec9A-DTR mice have been described in *Piva et al* 2012 [292].

Clec9A (DNGR-1) belongs to a family of C-type lectin-like molecules encoded on chromosome 6 of the mouse. The gene for Clec9A encodes a type II transmembrane protein of 264 amino acids with a single c-type lectin-like domain in its extracellular region, a cytoplasmic tail and a transmembrane region containing a potential signaling motif. Enhanced T cell-dependent antibody response was observed in mice when antigen is targeted to the Clec9A receptor using anti-Clec9A antibody [46]. Clec9A has also been shown to be required for the cross-presentation of necrotic cell-associated-antigens to CD8⁺ T cells [47]. Interestingly, Clec9A shows a more DC-restricted pattern of expression than other C-type lectins. Analysis of its expression on a panel of hematopoietic cells has shown that Clec9A is not expressed on monocytes, macrophages and T cells. Although NK cells express some mRNA for Clec9A, no surface protein is detectable. More importantly, the expression of Clec9A is negative

in the CD8⁻ lymphoid tissue cDCs and the monocyte-derived inflammatory DCs. Clec9A expression is limited to DCs, in particular the CD8⁺ cDCs and at lower levels, pDCs in spleen, lymph nodes and thymus. In addition, Clec9A is also expressed on CD11b⁻CD103⁺ cDCs of peripheral tissues including the small intestines and colon [46].

Clec9A expressed on mouse CD8⁺ DCs and human CD141⁺ DCs has been shown to function as a receptor in recognizing and binding to exposed actin filaments on dying cells, hence are categorized as a damage-cell associated molecular pattern (DAMP) receptor [46, 270, 271, 272]. This enables DCs to process and acquire pathogen-derived antigens from dying cells resulting in presentation of these antigens to T cells. In addition, Clec9A also regulates cross-presentation of dead cell-derived antigen via a Syk-dependent manner [271].

Clec4a4-DTR

The C-type lectin Clec4a4 (also known as dendritic cell inhibitory receptor, DCIR2), is expressed in DCs, specifically in CD8⁻ cDC subset in spleen, LN and PP. The restricted expression of Clec4a4 in CD8⁻ cDCs highlight its suitable use as a promoter in a DT-DTR cell ablation system, enabling the specific depletion of the intestinal mucosa counterparts of splenic CD8⁻ DCs identified as CD103⁺CD11b⁺ DCs. Clec4a4 contains an ITIM motif located at the cytoplasmic tail, suggesting the inhibitory properties of this lectin [254]. Clec4a4 is recognized by anti-33D1 antibody hence can be used to detect CD103⁺CD11b⁺ DCs. Clec4a4-DTR mice generated by our lab enabled us to study the consequence of depleting intestinal CD103⁺CD11b⁺ DCs in a DSS model of intestinal inflammation.

1.5 Aim of thesis.

In this thesis, we aim to elucidate the possible contributions of colonic CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DC subsets to innate mucosal immunity, specifically in their ability to regulate intestinal inflammation and maintenance of tissue immune homeostasis. To understand the mechanism of how colonic DCs control intestinal inflammation, the cross-talk between DCs, other immune cells and intestinal epithelial cells which forms an important barrier against the potentially hostile external environment will be studied.

2. MATERIALS AND METHOD

2.1 Animals

BALB/c background Clec9A-DTR mice were generated in our lab as described in *Piva et al* (2012) [292]. In brief, the generation of Clec9A-DTR mice involves the preliminary generation of genetically modified embryonic stem (ES) cells via electroporation of the bacterial artificial chromosome (BAC) where the DTR will be expressed under the control of the Clec9A promoter. BAC constructs consisting of a core expression cassette was constructed by Wee Nih Fam (SBS, NTU, Singapore). Details of the core expression cassette were listed in figure 2.1. These BAC-electroporated ES cells were then microinjected into blastocysts of donor mice and implanted into pseudo-pregnant females. The resultant chimeric offsprings will be crossed to a WT background to generate offsprings positive for the Clec9A-DTR BAC construct.

Figure 2.1. Design of BAC construct. The core expression cassette consists of the human DTR gene, 2A: cotranslation cleavage mediating linker, tdTomato: reporter gene, pA: polyadenylation site. The core expression cassette was placed at the initiation codon of the Clec9A gene.

The generation Clec4a4-DTR mice was generate by Dr Piotr Tetlak (SBS, NTU, Singapore) using a gene targeting approach (Figure 2.2). A gene construct consisting of a modified Clec4a4 gene with an IRES-DTR and selection marker PGK-NeoR was

inserted downstream after its stop codon within the 3' UTR of the Clec4a4 gene. Gene cassette was then introduced into BALB/c ES by electroporation followed by selection of transformed ES clones by Neomycin selection. Subsequent steps involving implantation of transformed blastocysts into pseudo-pregnant females and crossing of chimeric offsprings to obtain stable Clec4a4-DTR lines were carried out similarly to Clec9A-DTR. Both Clec9A-DTR and Clec4a4-DTR were viable and fertile, and no differences in phenotype were observed when compared to WT mice.

Figure 2.2. Design of targeting vector. The core expression cassette consists of a human DTR cDNA, followed by pA: polyadenylation sequence and neomycin resistance gene for selection. The successful knock-in of the core expression cassette results in its insertion after the stop codon of exon 6 in the Clec4a4 gene.

ROR γ t-GFP and CX3CR1-GFP mice were generated by Dr. D Littman (New York) and were kindly provided by Dr Florent Ginhoux from SIgN, A*STAR, Singapore. All breedings and experiments were carried out in the NTU animal facility under specific pathogen-free conditions.

2.2 Isolation of DCs from mesenteric lymph nodes and Peyer's patches

Peyer's patches and mesenteric lymph nodes were carefully excised, chopped and digested in serum-free IMDM containing 100 μ g/ml collagenase D (Roche) with shaking at 370 rpm, 37°C, for 60 mins. Digested pieces were passed through a nylon strainer (100 μ m) to remove tissue debris. Cell suspensions containing released cells were centrifuged and gradient-purified to obtain dendritic cells and macrophages as

described in *Ruedl et al* 2009 [268]. In brief, cell pellets obtained after collagenase step were resuspended in 4ml resuspension buffer (3ml Hanks buffer + 1ml OptiPrep). 4ml of gradient buffer (4.1ml Hanks buffer + 1ml OptiPrep) was then gently overlayed on the resuspension buffer containing the single cells. Finally, 3.5ml of Hanks buffer was gently overlayed on the gradient buffer, resulting in a preparation containing an upper and lower interface. Preparation was then centrifuged at 600g for 20 minutes with brakes removed. Cells containing an enrichment of DCs and macrophages were isolated from the upper interface using a pipette tip and transferred into a separate tube. 10ml of sterile Hanks buffer was added to the cell suspension. Diluted cell suspension was then centrifuged at 350g for 10 minutes to recover isolated DCs and macrophages.

2.3 Isolation of colonic LP leukocytes

Colons were removed (excluding the caecum), longitudinally opened and flushed using cold 2% PBS to remove luminal contents. Longitudinally-opened colons were then cut into 1 cm pieces and washed in Hanks' balanced salt solution (HBSS) containing 1 mM DTT at 37°C with shaking at 370rpm for 20 mins. To remove epithelium, colons were incubated in Hanks buffer containing 1.3mM EDTA at 37°C, with shaking at 370rpm, for 60mins (Fresh EDTA solution every 20mins). EDTA-treated colon pieces were soaked in serum-free IMDM for 10mins, and then blotted on paper towels to remove EDTA. Colons were then incubated in serum-free IMDM containing 100µg/ml collagenase D (Roche Applied Science, Basel, Switzerland) with shaking at 37°C, 370rpm, for 90mins. Digested colonic pieces were then passed through a cell strainer. The leukocyte population containing the DCs and macrophages was enriched by a 70%/40% Percoll gradient which was centrifuged at 2800 rpm for 10 minutes. Total colonic leukocytes were bound at the 70%/40% interface and harvested using a pipette-tip. Cells were kept on ice till use.

2.4 Isolation of colonic intraepithelial leukocytes (IEL).

Colons were dissected, opened longitudinally and washed in cold PBS to remove fecal contents. Colonic IECs were isolated from colonic mucosa through shaking in 25ml PBS containing 2% FCS and 1.3mM EDTA, 270rpm, 37°C for 1 hour. Released epithelial cells were collected from the EDTA solution at every 20 mins through centrifugation at 350g. Fresh EDTA solution was added to the tube after each collection of IECs. Resulting IEC pellets were pooled at the end of the isolation step,

and washed in serum-free IMDM. Washed IECs were transferred to 5ml serum-free IMDM containing 0.1 mg/ml collagenase D (Roche Applied Science, Basel, Switzerland) and shaken at 370rpm, 37°C for 20mins. Digested IECs were then passed through a wire mesh and cells were collected by centrifugation at 350g. IELs were enriched using a 44%/67% Percoll gradient centrifuged at 600g for 10 minutes.

2.5 In vivo ablation of DC subsets

DCs were *in vivo* ablated in DTR mice after administration of DT. 20ng/g body-weight of DT (Sigma-Aldrich) in PBS containing 1% mouse serum was injected intraperitoneally to DTR mice. For analysis of depletion efficiency, CX3CR1-GFP^{+/−} DTR mice were injected at day -1 and sacrificed at day 0. MLNs and colons were then removed and processed for isolation of single cell suspensions. Cells were blocked of their Fc-receptors followed by antibody staining using: PEcy7-conjugated anti-CD45.2 (1:600), PE-conjugated anti-CD11c (1:600), Pacific Blue-conjugated anti-MHCII (1:800), APC-conjugated anti-CD64 (1:400), PerCyP Cy5.5-conjugated anti-CD103 (1:600) and APC cy7-conjugated anti-CD11b (1:600) for 20 minutes at 4°C. FACS analysis of immunostained cells were carried out using the 5-lasers BD Fortessa X-20.

2.6 Induction of DSS-induced colitis

Age-matched female 8 weeks old WT and DTR mice (n > 4) were given 2% DSS (50,000 Da, MPBiomedical, Santa Ana, CA, USA) dissolved in sterile distilled water. Mice were supplied *ad libitum* with fresh DSS solution every 3 days for 7 days, after which the DSS solution was replaced with sterile drinking water for another 7 days. Daily records of DSS consumption were taken to ensure mice receive sufficient DSS. In DT-ablated mice, 20ng/g b.w.t of DT was injected intraperitoneally, 24 hours before start of DSS schedule. Mice were then given DT injection subsequently at days 0, 3, 6, 9 and 12. Mice were assessed for severity of colitis throughout the entire DSS regimen and sacrificed at day 14.

2.7 Evaluation of colitis severity

Assessment of colitis severity was evaluated through (1) Daily monitoring of body-weight loss. (2) Presence of rectal bleeding. (3) Detection of fecal occult blood (hematochezia) using Hemocult SENSE (Beckman Coulter, Brea, CA) (4) Colon shrinkage - length of colon taken between ileocaecal junction and the proximal rectum.

Colons were measured on a non-absorbent surface, taking care not to stretch the tissue. (5) Hematoxylin and Eosin (H.E) staining of distal colon for histological analysis of mucosal damage and extent of immune cell infiltration in tissue. (6) Percentage of inflammatory neutrophils and monocytes. (7) Epithelial permeability assay using the tracer molecule FITC-dextran (4 kDa, Sigma-Aldrich, St. Louis, USA).

2.8 Hematoxylin and Eosin staining of mouse colons.

Colons were removed (excluding the caecum), longitudinally opened and washed in cold PBS 2% FCS to remove luminal contents. Colons were then fixed in 4% formaldehyde at 4°C overnight. After fixation, colons were then swiss-rolled and then dehydrated in 80% 90% 100% ethanol for 2 hours, 2 hours and 4 hours respectively. Dehydrated swiss-rolled colons were then transferred into 1:1 mixture of ethanol : xylene for 30 mins followed by pure xylene for 3 hours. Colons were then soaked in molten paraffin overnight and then set in moulds for sectioning. Colon sections of 7 µm thickness were obtained using microtome, and subsequently stained with hematoxylin and eosin. In brief, cut sections were deparaffinized in xylene and rehydrated in 95% and 70% alcohol at 5 minutes each. Sections were then rehydrated in distilled water briefly and then differentiated in 1% acid alcohol for 30 seconds. Slides were then washed in running tap water for 1 minute followed by a bluing step in 0.2% Scotts tap water for 1 minute. Slides were then washed in running tap water for 5 minutes and dipped 10X in 95% ethanol. Tissues were counterstained in eosin-phloxine B solution for 10 seconds following dehydration through 95% and 100% ethanol for 5 minutes each. Slides were cleared in 2 changes of xylene for 5 minutes each and mounted using a xylene based mounting medium.

2.9 Periodic Acid Schiff staining

Visualisation of total mucin distribution on colonic mucosa was carried out by PAS staining (Periodic Acid Schiff (PAS) Stain Kit, Abcam). PAS staining was performed on acetone-fixed 8µm swiss-rolled colon cryosections as stated by manufacturer's protocol. Stained sectioned were analysed using a brightfield microscope (Nikon Eclipse 80i, Nikon Instruments, Melville, NY, USA) at 20x magnification. Mucins and goblet cells were stained red, colonic tissues were counter-stained green.

2.10 Detection of fecal occult blood.

Fecal blood was detected by using a commercially available kit, Hemocult SENSE (Beckman Coulter, Fullerton, CA). Development of blue coloration tests positive for presence of fecal blood. The scoring of color intensity in brief; 0: No blue color, +1: Barely detectable blue, +2: Faint blue color, +3: Distinctly blue, +4: Intense blue or wider area of blue than 3.

2.11 FITC-dextran Intestinal Epithelium Permeability Assay.

To assess the intestinal membrane permeability of DSS-treated mice, a FITC-conjugated dextran macromolecule (4 kDa, Sigma-Aldrich, St. Louis, USA) was used as a probe. Mice were given 2% DSS freshly supplied every 3 days for 7 days, followed by water thereafter. FITC-dextran prepared at a concentration of 20mg/ml was introduced into DSS-treated mice via oral-gavage with each mice given 60mg/100g body weight of FITC-Dextran. Feed pellets and drinking water were removed from cages of DSS-treated mice at 24h and 2h respectively prior to oral-gavage of FITC-dextran. Blood was extracted from individual mice after 4 hours oral gavage, to measure the concentrations of serum FITC-dextran at days 0, 4 and 10 of DSS regiment. Serum collected from whole blood was diluted 5x prior measurement of FITC-dextran using the spectrophotometer TECAN Infinite 200 PRO multimode readerTM set to 488nm excitation and 535nm emission wavelengths.

2.12 Antibody staining for FACs analysis

Single-cell suspensions of the tissues to be examined were stained with a combination of different monoclonal antibodies (mABs) diluted in PBS containing 2% FCS. Prior to staining with desired antibodies, cell isolations were first blocked of their Fc-receptors using the antibody anti-CD16/CD62. Cells cell then stained with the desired antibodies for 20 mins, washed and resuspended in PBS 2% FCS. In the case of intracellular Foxp3 staining, cells were first stained for extracellular markers and then fixed and permeabilised using Foxp3 Fixation and Permeabilisation Kit (eBioscience) prior to staining using anti-Foxp3. FACs analysis was performed on a FACSCaliber (BD Biosciences), or 5-laser BD Fortessa X-20. FACs data was analysed by Flojo software (TreeStar Inc).

2.13 RNA preparation from sorted colonic dendritic cell subsets and microarray analysis.

Colonic dendritic cell subsets were sorted on BD FACs Aria using the antibodies FITC-conjugated anti-MHCII, PE-conjugated anti-CD11c, PercypCy5.5-conjugated anti-CD11b and APC-conjugated anti-CD103. Total of 3 individual cell isolations, each from a pool of 8 colons per group (Steady-state vs Day 4 2% DSS-treatment) were sorted into a solution containing 10% BSA. Cells were gated on MHCII⁺CD11c^{hi} population and sorted based on CD103 and CD11b expression - Subset 1: CD103⁺CD11b⁻. Subset 2: CD103⁺CD11b⁺. Subset 3: CD103⁻CD11b⁺. Total RNA was then isolated from individually sorted populations using the Arcturus Picopure RNA Isolation Kit (Life Technologies). The RNA Integrity Number (RIN) was calculated by using Agilent Bioanalyzer and individual samples yielded a value of ≥ 9.4 . ssDNA was prepared from 5ng of extracted RNA using the WT Ovation Pico RNA Amplification and Encore Biotin (NuGEN). Amplified ssDNA was then fragmented and labelled according to manufacturer's instructions to yield SPIA cDNA upon which 5µg were used for hybridisation to Affymetrix GeneChip® Mouse Gene 1.0 ST Arrays for 17 hours at 45°C. The arrays were then washed and stained using the Affymetrix Fluidics Station 450 and scanned using GeneChip Scanner 3000. Data of differential gene expression was analysed using Expression Console and comparisons of gene expression was carried out according to Affymetrix. Gene expressions were normalized using RMA/quantile normalization [267], and differential gene analysis were performed using limma [268]. Analysis of gene expression levels, comparisons of transcriptomes between subsets resulting in a generation of PCA plot were performed in R 2.15.2/bioconductor, through Pipeline Pilot. A TM4 program was used to generate heatmaps of differential gene expressions [269].

2.14 Extraction of colonic epithelial cell RNA and microarray analysis

IECs were harvested from distal (last 1/3) colonic epitheliums of steady state and day DSS-treated colons by agitation in PBS containing 1.3mM EDTA, at 37°C for 1hour. Released epithelial cells were collected at every 20 minute interval by centrifugation and fresh EDTA solution added to the distal colons after each interval. Collected epithelial cell fractions were stored on ice and pooled together after 1 hour. To extract total mRNA, epithelial cell pellets were lysed in Trizol (Life Technologies) and

chlorofoam was added to the homogenate. The mixture was then centrifuged at 12000g for 15mins at 4°C resulting in separation of mixture into both upper aqueous phase and a red lower phase. Total RNA was then collected from the upper colorless aqueous phase and precipitated in isopropyl alcohol and resuspended in RNase-free water. The freshly collected RNA was then digested with DNase1 to remove contaminating genomic DNA and subsequently purified using chlorofoam-isopropanol precipitation. Following a wash step in 75% ethanol, RNA pellet was then resuspended in RNase-free water and stored at -80°C.

RNA was prepared for microarray analysis using Low Input Quick Amp Labeling kit, One-Color (Agilent p/n 5190-2305) resulting in generation of labeled cDNA. In brief, 100ng of RNA was first *in vitro* transcribed into cDNA using T7 RNA polymerase and oligo-dT primers. Transcription of RNA with T7 RNA polymerase using the Low Input Quick Amp Labeling kit generated cyanine 3-CTP labeled cRNA which 600ng was used in hybridization onto Agilent SurePrint G3 Human GE 8x60K Microarray for 17 hours at 65°C, agitation at 10 rpm in Agilent hybridisation oven. Microarray chip was then scanned on Agilent High Resolution Microarray Scanner (C-model). Data generated was extracted by Agilent Feature Extraction Software (V10.7.1.1).

2.15 Quantitation of colonic epithelial gene-expression using semi-quantitative PCR

Total RNA was isolated from distal colonic epithelium as described in section 2.14. Total RNA (5ug) was converted to cDNA using Superscript reverse transcription (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions. To quantitate target gene expression, 8ng of cDNA were used per PCR reaction (GoTaq® Flexi DNA Polymerase, Promega). Gene expressions were normalized to *Hprt*. PCR amplification was carried out on a Thermocycler T3000 (Biometra GmbH, Goettingen, Germany). 10ul of PCR reaction (50µl) were loaded on 2% agarose gel to visualize amplified DNA bands. Images of amplified fragments were captured in a UV-transilluminator system. Primer sequence of genes analysed were listed in table 2.3.

Gene	Primer sequence	Fragment size (Bp)
<i>Reg3β</i>	Fwd: GGCTTCATTCTTGTCTCCA Rev: CCCTTGTCCATGATGCTCTT	332
<i>Reg3γ</i>	Fwd: CCTGATGCTCCTTTCTCAGG Rev: ACTCCCATCCACCTCTGTTG	314
<i>S100a8</i>	Fwd: TCGTGACAATGCCGTCTGAA Rev: TTTGTGAGATGCCACACCCA	260
<i>S100a9</i>	Fwd: GGCCAACAAAGCACCTTCTC Rev: CACGTGGGTTGTTCTCATGC	302
<i>Relmβ</i>	Fwd: AGCCTAAGACGATCTCCTGC Rev: TTAAGCCATTTCGGCAGCAGC	191
<i>Il18bp</i>	Fwd: GAGCACAGGAACACAAGCAC Rev: GGGCCTGCTGATCTGGATAC	221 and 428
<i>Il22ra1</i>	Fwd: TTCCATGGGTTTCCTCGTCG Rev: CTTGGGAGTACTGGATGGGC	200
<i>Il22ra2</i>	Fwd: CCGGTCGTGACTATAACCCG Rev: CGCAGTAGCTGGAATGAGGT	229
<i>Ido1</i>	Fwd: CTGTATGAGGGGGTCTGGGA Rev: GACTGGGGGAGCTGACTCTA	207
<i>IfnyR</i>	Fwd: GTGGTTGCTCCTCTTACCGTC Rev: GCCTCCAGAGCCTTTGTTTCT	302
<i>Hprt</i>	Fwd: GCTGGTGAAAAGGACCTCT Rev: CACAGGACTAGAACACCTGC	249

Table 2.1. List of primers used to quantitate gene expression using semi-quantitative PCR analysis.

2.16 Fluorescence staining of MUC2, REG3 γ and REG3 β

Colonic tissues were excised, opened longitudinally and bowel contents were removed cleanly using a pair of forceps. Embedding medium (O.C.T Tissue-Tek, Fisher Scientific) was then applied to the lumen side. Colons were then swiss-rolled and transferred into moulds containing embedding medium and snapped frozen in dry-ice. Frozen tissues were allowed to set overnight at -80 °C. Colons were sliced to 8 μ m thickness using a microtome (Leica RM2265, Leica Microsystems, Wetzlar, Germany), mounted on glass slides and allowed to dry for 1 hour. Prior to the staining procedure, sections were fixed in acetone for 10 minutes and rehydrated in PBS for 2 minutes. Rehydrated sections were then blocked in PBS containing 10% FCS and Fc-block, for 30 minutes at 4 °C. Polyclonal primary antibodies of either rabbit anti-MUC2, rabbit

anti-REG3 γ or rabbit anti-REG3 β were diluted 1:100 in blocking buffer containing 0.05% Triton X-100. Sections were stained overnight at 4 °C, washed in PBS containing 2% FCS the next day for 1 hour. Sections were then stained with secondary antibody (anti-rabbit FITC 1:500), incubated for 1 hour at 4 °C followed by a wash-step in 2% PBS for another 1 hour. Sections were then mounted with anti-fade mountant (VECTASHIELD[®], Vector Laboratories, Burlingame, CA, USA) containing the nuclear staining dye, DAPI. Sections were then analysed under confocal microscopy (Zeiss LSM510 META inverted, Goettingen, Germany) acquired with identical settings using Plan-Apochromat 20x/0.8 lens, to compare expression levels of MUC2, REG3 γ and REG3 β . Images were acquired and processed using the ZEN Imaging Software (Zeiss).

2.17 Fluorescence staining of Clec9A⁺ and Clec4a4⁺ colonic DC subsets.

Colons were swiss-rolled and cryofrozen in embedding medium, sliced to 8 μ m thickness using a microtome, acetone-fixed and blocked as described in section 2.16. Monoclonal PE-conjugated anti-Clec9A, APC-conjugated anti-33D1 (Clec4a4) and FITC-conjugated anti-CD11c antibodies were diluted (1:100) in blocking buffer containing 10% FCS and 0.1% Triton X-100. Cryosections were stained with antibody mix overnight at 4 °C. Sections were then washed in 2% PBS for 1 hour and mounted in anti-fade mountant (VECTASHIELD[®], Vector Laboratories, Burlingame, CA, USA). In the case of colons obtained from CX3CR1-GFP tg-mice, mice were perfused with PBS containing 2% PFA and 30% sucrose prior to dissection of colons. Sections were analysed by confocal microscopy (Zeiss LSM510 META inverted, Goettingen, Germany) with identical settings using Plan-Apochromat 20x/0.8 lens. Images were acquired and processed using the ZEN Imaging Software (Zeiss).

2.18 Intracellular IL-22 and IFN- γ staining.

To analyse IL-22⁺ immune cells of colonic LP, colons were dissected and washed in ice-cold PBS to remove luminal contents. Subsequently, total colonic LP leukocytes were isolated as previously described in section 2.3. Isolated cells were then stimulated in IMDM containing 10⁻⁴ mM of phorbol 12-myristate 13-acetate (PMA) and ionomycin, and 2% FCS for 3 hours followed by 10 μ g/ml Brefeldin A for another 2 hours. Stimulated cells were labeled for extracellular markers followed by fixing in 2% PFA at room temperature for 1 hour, and permeabilisation in 0.5% saponin.

Permeabilised cells were then labeled with APC-conjugated anti-IL-22 and analysed by FACSCaliber (BD Biosciences).

To analyse IFN- γ ⁺ CD8⁺ and IFN- γ ⁺ CD4⁺ colonic LP and IELs T-cells, isolated LP and colonic epithelial leukocytes (methods of isolation described in section 2.3 and 2.4 respectively) were stimulated in IMDM containing 10⁻⁴mM PMA/ionomycin and 2% FCS for 2 hours followed by the 10 μ g/ml Brefeldin A treatment for another 2 hours. Stimulated cells were labeled for extracellular markers followed by fixation in 2% PFA at room temperature for 1 hour, and subsequently permeabilised in 0.5% saponin. Permeabilised cells were then labeled with PE-conjugated anti-IFN- γ and analysed by FACs analysis (5-laser Fortessa X-20 BD, Biosciences).

2.19 Treatment with TLR9 ligands (Immunostimulatory DNA, ISS-ODN)

To induce strong IFN- γ expression in WT and Clec9A-DTR mice, 10 μ g of immunostimulatory oligonucleotide ISS-ODN (5'-TGACTGTGAACGTTTCGAGATGA-3') were intra-peritoneally introduced into experimental mice at day 0 and 4. Mice were given 2% DSS from day 0 to day 4. Clec9A-DTR mice were treated with 20ng/g body-weight DT at day -1 and 4. Levels of IFN- γ were measure in serums of experimental mice at day 4 using ELISA, to check the efficacy of induction. The use of ISS-ODN to stimulate IFN- γ production resulting in upregulation of IDO1 in colonic epithelium has been described in Ciorba *et al*, 2010[339].

2.20 Statistical analysis

Statistical significance was determined by unpaired Student *t*-test using commercially available software (PRISM). Values of $p < 0.05$ were considered statistically significant. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

2.21 Computer softwares

Flow cytometric data were analysed using FlowJo 7.6.1 software (TreeStar Inc, Ashland). Charts, dotplots and statistical analysis were done using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA, USA). Confocal images in LSM format were analysed using AimImageBrowser (Carl Zeiss MicroImaging, GmbH). Gene

ontology analysis of highly enriched genes from sorted MHCII^{hi}CD11c^{hi} cell subsets was carried out using the on-line bioinformatics tool AmiGO 2 version 2.2.0 (Gene database: PANTHER GO biological process complete, released 2015-08-06).

3. RESULTS

3.1 Defining distinct subsets of CD11c^{hi}MHCII⁺ myeloid cells in mouse colon.

Mouse colonic CD11c^{hi}MHCII^{hi} myeloid cells can be characterized into three distinct subsets; CD103⁺ CD11b⁻, CD103⁺ CD11b⁺ and CD103⁻ CD11b⁺. In this section, we illustrate the different subsets of mouse colonic CD11c^{hi}MHCII^{hi} myeloid cells (3.1.1). To enable us to further classify them, a genome-wide transcriptional profile was done (3.1.2). Results from the genome-wide transcriptional profiling allows not only the characterization of CD11c^{hi}MHCII^{hi} subsets, but also identification of two specific lectins; Clec9A (DNGR-1) and Clec4a4 (DCIR2) which are expressed respectively on CD103⁺ CD11b⁻, CD103⁺ CD11b⁺ colonic DCs, hence allowing their classification based on these markers.

3.1.1 FACs analysis of CD11c⁺MHCII⁺ myeloid cell subsets in mouse colon and mesenteric lymph nodes.

We aim to characterize distinct subsets of mouse MLN and colonic CD11c⁺MHCII⁺ myeloid cells using FACs analysis. Whole steady-state MLN and colonic tissues were dissected from 8 week old BALB/c mice and prepared for isolation of single cell suspensions as described in materials and methods section. Table 3.1 summarized the list of markers used for identification of mouse MLN and colonic CD11c⁺MHCII⁺ myeloid cell subsets.

	Cell subset	Antibody combination
Colonic CD11c ^{hi} MHCII ^{hi}	CD103 ⁺ CD11b ⁻ LP cDCs	CD45 ⁺ CD103 ⁺ CD11b ⁻ CD11c ^{hi} MHCII ^{hi}
	CD103 ⁺ CD11b ⁺ LP cDCs	CD45 ⁺ CD103 ⁺ CD11b ⁺ CD11c ^{hi} MHCII ^{hi}
	CD103 ⁻ CD11b ⁺ LP macrophage	CD45 ⁺ CD103 ⁻ CD11b ⁺ CD11c ^{hi} MHCII ^{hi}
Colonic CD11c ^{int} MHCII ^{hi}	CD103 ⁻ CD11b ⁺ LP macrophage	CD45 ⁺ CD103 ⁻ CD11b ⁺ CD11c ^{int} CD64 ⁺ MHCII ^{hi} CX3CR1-GFP ^{hi}
	CD103 ⁻ CD11b ⁺ Lp monocytes	CD45 ⁺ CD103 ⁻ CD11b ⁺ CD11c ^{int} CD64 ^{int} MHCII ^{hi} CX3CR1-GFP ^{int}
MLN CD11c ^{hi} MHCII ^{hi}	CD103 ⁺ CD11b ⁻ migratory cDCs	CD45 ⁺ CD103 ⁺ CD11b ⁻ CD11c ^{hi} MHCII ^{hi}
	CD103 ⁺ CD11b ⁺ migratory cDCs	CD45 ⁺ CD103 ⁺ CD11b ⁺ CD11c ^{hi} MHCII ^{hi}
MLN CD11c ^{hi} MHCII ^{int}	CD8 ⁺ CD11b ⁻ resident cDCs	CD45 ⁺ CD8 ⁺ CD11b ⁻ CD11c ^{hi} MHCII ^{int}
	CD8 ⁻ CD11b ⁺ resident cDCs	CD45 ⁺ CD8 ⁻ CD11b ⁺ CD11c ^{hi} MHCII ^{int}

Table 3.1. List of markers used for FACs analysis of colonic and MLN myeloid cell subsets.

FACs analysis of percoll-gradient purified LP cell suspensions yielded a major population of CD45⁺ cells of hematopoietic origin (figure 3.1). CD45⁺ cells were then gated to analyze myeloid cells comprising of 1) MHCII^{hi}CD11c^{hi} and 2)MHCII^{hi}CD11c^{int}. MHCII^{hi}CD11c^{hi} cells comprised of two distinct subsets of CD103⁺ cDCs; CD103⁺CD11b⁻ and CD103⁺CD11b⁺, and a third mononuclear phagocyte population CD103⁻CD11b⁺. MHCII^{hi}CD11c^{int} cells on the other hand, comprised of CD64⁺CX3CR1^{hi} monocyte-derived macrophages and CD64^{int}CX3CR1^{int} monocytes.

In optiprep-gradient enriched MLN cells, CD45⁺ cells comprised of CD11c⁺ cells that can be further segregated based on MHCII^{hi} and MHCII^{int}. CD11c⁺MHCII^{hi} cells are migrating cDCs arising from from small intestine and colonic lamina propria [273], and they comprised of CD103⁺CD11b⁻ and CD103⁺CD11b⁺. In the CD11c⁺MHCII^{int} gate, resident MLN cDCs were present and they comprised of CD8⁺CD11b⁻ and CD8⁻CD11b⁺ cDCs.

Figure 3.1. Distinct CD11c⁺MHCII⁺ myeloid cell subsets in mouse colonic LP and MLN. Flow-cytometric analysis of CD45⁺ colonic LP and MLN cell suspensions. (A) Colonic LP CD11c^{hi}MHCII^{hi} cells (gate 1) consists of CD103⁺CD11b⁻, CD103⁺CD11b⁺ cDCs and CD103⁻

CD11b⁺ MNP, while CD11c^{int}MHCII^{hi} cells (gate 2) comprised of CX3CR1^{hi}CD64⁺ steady-state macrophages as well as CX3CR1^{int}CD64^{int} tissue monocytes. Numbers reflect the percentages of gated cell populations. (B) Pooledoptiprep gradient-purified MLN from 3 mice: CD11c^{hi}MHCII^{hi} cells (gate 3) consisting of migratory lamina propria CD103⁺CD11b⁻ and CD103⁺CD11b⁺ cDCs while CD11c^{hi}MHCII^{int} (gate 4) consisting of classical lymphoid CD8⁺CD11b⁻ and CD8⁺CD11b⁺ cDCs. Numbers reflect the percentages of gated cell populations. (C) Percentages and absolute numbers of gated cell populations in colonic LP (*n*=3) and pooled gradient-purified MLN cells from 3 mice.

FACs analysis of colonic CD45⁺MHCII⁺CD11c⁺ leukocytes, clearly revealed multiple subsets of cDC and other mononuclear phagocytes. cDCs can be distinguished from other mononuclear phagocytes by their high expression of CD11c and CD103 while lacking in expression of CX3CR1 and the IgG receptor FcγR1 (CD64). These CD11c^{hi}MHCII⁺CD103⁺ cDCs can be divided as CD103⁺CD11b⁻ and CD103⁺CD11b⁺ and has been described as migratory cDCs of the LP [276]. A third subset in the CD11c^{hi}MHCII⁺ population (gate 1) is the CD103⁻CD11b⁺ subset which lacks the monocyte marker CD64. Characterization of this subset, whether it can be classified as cDC or macrophage is still controversial [277-280] and as will be discussed in section 3.1.2, our transcriptional profiling analysis suggests this subset can be delineated as macrophages.

CD64⁺ mononuclear phagocytes which includes the CX3CR1^{hi} macrophage subset (Figure 3.1, gate 2) do not express CD103 and have been described as tissue resident macrophages [274-275]. A population in the CD64⁺ mononuclear phagocytes also include the CX3CR1^{int} monocytes which have been described to express F4/80, arise from Ly6C^{hi} monocytes and are precursors to various tissue-resident macrophages [274-275].

3.1.2 Genome-wide transcriptome analysis of sorted colon CD11c^{hi}MHCII^{hi} myeloid cell subsets.

In this thesis, we aim to study the role of different dendritic cell subsets in gut immunity. To study the transcriptional profiles of the three CD11c^{hi}MHCII^{hi} myeloid cell subset, we sorted CD11c^{hi}MHCII^{hi} cells isolated from colons of 8 week old female BALB/c control and DSS-treated mice (Day 4). In the DSS-treated mice group, 3% DSS was supplied in drinking water *ad libitum* to induce mild colitis, hence enabling the comparison of transcriptional profiles of the three myeloid cell subsets in an acute inflammatory environment. Isolation of colonic CD45⁺ leukocytes (pool of 8 colons per control and DSS-treated group, each group done in triplicate) was performed as stated in materials and method section. Gating strategy to sort CD103⁺CD11b⁻ CD103⁺CD11b⁺ CD103⁻CD11b⁺ myeloid cells has been illustrated in figure 3.2.

Figure 3.2. Transcriptome analysis of sorted colonic CD45⁺CD11c^{hi}MHCII⁺ myeloid cell subsets. (A) Gating strategy of sorted colonic CD11c^{hi}MHCII⁺ myeloid cell subsets. CD45⁺ cells were gated to reveal CD11c^{hi}MHCII⁺, which were then sorted based on 1) CD103⁺CD11b⁻ 2) CD103⁺CD11b⁺ 3) CD103⁻CD11b⁺ populations. Cell isolations were obtained from a pool of 8 control and DSS-treated colons (day 4), with each group performed in triplicate. Following cell isolation, RNA was extracted from each population and analysed in a microarray. (B) Transcript heatmaps displaying ~640 genes that were differentially expressed by at least 2 folds. (Red: upregulated; green: down regulated). Clustering was

performed using Pearson's correlation and complete linkage. Heatmap was z-score normalized by row. (C) XY plot of the first two components of a principal component analysis (PCA) of all six groups (SS 1-3 and DSS 1-3) (D) Heat map showing differential expression of selected genes involved in DC development and function. SS: Steady state condition. DSS: Day 4 3% DSS-treated mice.

To compare transcription profiles of the 3 mouse colonic CD11c^{hi}MHCII^{hi} myeloid cell subsets in steady-state as well as in DSS-induced inflammatory environment, hierarchical clustering of entire transcriptome was done using Pearson correlation and complete linkage. In an inflammatory setting (day 4 DSS), all three subsets showed clear clustering with subset 1 (CD103⁺CD11b⁻) showing the most difference in terms of transcription profile when compared to the other subsets. Subset 2 (CD103⁺CD11b⁺) and subset 3 (CD103⁻CD11b⁺) showed higher degree of similarity but can be delineated as distinct subsets based on clear clusterings of their transcriptome profiles marked in the PCA plot (Figure 3.2C). In steady-state, CD103⁺CD11b⁻ subset showed a clear clustering of transcriptome profile while CD103⁺CD11b⁺ and CD103⁻CD11b⁺ subsets showed more overlaps (Figure 3.2C).

Clear clustering of CD103⁺CD11b⁻ subset suggests the unique identity of this cell population. Observing the genes highly expressed in CD103⁺CD11b⁻ subset revealed transcription factors linking to regulation of interferons such as *Irf8*, *Irf5* and inhibitor of DNA-binding *Id2* and the transcription factor *Batf3*. Other genes includes the c-type lectin *Clec9A*, adhesion molecule *Cd24*, FMS-like tyrosine kinase 3 *Flt3*, chemokine receptor *Xcr1* and the integrin *Itga2* (CD49B) (Figure 3.2D). The expression of *Batf3* [281], *Irf8* [282] and *Id2* [283] has been shown to be critical key transcription factors needed for CD103⁺CD11b⁻ dendritic cell differentiation program. Other markers such as *Flt3* [284] *CD24* [285] and *Clec9A* [286] are typically expressed in dendritic cells. From the genes mentioned above that were found to be highly expressed in this population, we classify CD103⁺CD11b⁻ subset as bona fide dendritic cells. CD103⁺CD11b⁺ subset expressed some markers similar to CD103⁺CD11b⁻ DCs, albeit at lower levels such as *Flt3*, *Irf5* and *Id2*. Other markers typically expressed by myeloid cells were also detected in CD11b⁺CD103⁺ subset which includes granulocyte-macrophage colony stimulating factor 2 receptor (*CSF2rb2*), triggering receptor expressed on myeloid cells 1 (*Trem-1*), macrophage galactose N-acetyl-galactosamine specific lectin 2 (*Mgl2*), signal-regulatory proteins α and β (*Sirpa*, *Sirpb1a*, *Sirpb1b*), different lectins (*Clec4a1*, *Clec4d*, *Clec4d*, *Clec4a4*, *Clec10a*) and

Mmp12 (Figure 3.2D). Hence, our data strongly suggest, CD103⁺CD11b⁻ and CD103⁺CD11b⁺ can be classified as bona fide cDCs. The controversial CD103⁻CD11b⁺ subset, whether it can be classified as cDC or macrophage, expressed markers associated with macrophage identity. These markers include macrophage transcriptional factor *MafB*, as well as other macrophage-related markers such as *F4/80* (*Emr1*), *Cd68*, *Cd14*, *Tlr4*, *Lamp1*, mannose receptor (*Mrc1*), MP scavenger receptor (*Msr1*), chemokine receptor *CX3CR1*, matrix metalloproteinase (*Mmp13*, *Mmp14*) and complement receptors (*C5ar1* and *C3ar1*) (Figure 3.2D).

In characterizing all three subsets based on markers identified from our microarray, our data strongly suggests CD11c^{hi}MHCII^{hi} myeloid cells of the colon can be characterized as bona fide DCs - 1) CD103⁺CD11b⁻, 2) CD103⁺CD11b⁺ DCs, and a third CD103⁻CD11b⁺ subset resembling a distinct macrophage-related cell population.

We predicted major changes in transcription profiles between DC subsets isolated in steady state and inflammation. However, our microarray analysis did not reveal significant differences between DC subsets from the two environments. This suggests the early time point of DSS treatment may not be sufficient to induce a more pro-inflammatory transcription profile in different DC subsets.

To gain a further insight of the possible functions of these sorted DC and macrophage subsets, gene ontology analysis of enriched genes was carried out using AmiGO2 (Panther-GO biological processes complete) on-line tool. In this analysis, 25% of the highest expressing genes (fold expression: 8.04-12.32, total 381 genes) from all three sorted cell subsets from steady-state and day 4 DSS treated colons were included. The possible functions of these MHCII^{hi}CD11c^{hi} subsets were illustrated in table 3.2 (steady-state) and table 3.3 (day 4 DSS treated), and their highest ten possible functions derived from ontogenic analysis were ranked accordingly by the highest percentage of gene hits in the database.

CD103 ⁺ CD11b ⁻ DCs (Steady-state)					
Gene ontology (Biological process)	Total number of genes in database	Number of gene hits	PANTHER over-representation test score	+/-	P-value
Regulation of B cell differentiation.	25	6	>5	+	2.15E-02
Glycosphingolipid metabolic process	32	7	>5	+	5.96E-03
Positive regulation of TNF superfamily cytokine production.	60	9	>5	+	3.68E-03
Positive regulation of TNF production	59	8	>5	+	3.31E-02
Ceramide metabolic process	60	8	>5	+	3.74E-02
Sphingolipid metabolic process	97	12	>5	+	3.71E-04
Regulation of TNF superfamily cytokine production	104	12	>5	+	7.78E-04
Leukocyte chemotaxis	87	10	>5	+	3.04E-03
Regulation of B cell activation	107	12	>5	+	1.05E-03
CD103 ⁺ CD11b ⁺ DCs (Steady-state)					
Gene ontology (Biological process)	Total number of genes in database	Number of gene hits	PANTHER over-representation test score	+/-	P-value
Glycosphingolipid catabolic process	11	5	>5	+	7.01E-03
Glycolipid catabolic process	12	5	>5	+	1.07E-02
Fc receptor signaling pathway	13	5	>5	+	1.58E-02
Ceramide catabolic pathway	14	5	>5	+	2.26E-02
Sphingolipid catabolic process	20	6	>5	+	6.21E-03
Membrane lipid catabolic process	22	6	>5	+	1.07E-02
Glycosphingolipid metabolic process	32	8	>5	+	3.62E-04
Positive regulation of TNF superfamily cytokine production	60	11	>5	+	2.69E-05
Regulation of IL-1 production	47	8	>5	+	6.43E-03
CD103 ⁻ CD11b ⁺ Macrophages (Steady-state)					
Gene ontology (Biological process)	Total number of genes in database	Number of gene hits	PANTHER over-representation test score	+/-	P-value
Fc-gamma receptor signaling pathway	7	5	>5	+	8.15E-04
Fc receptor signaling pathway	13	7	>5	+	1.54E-05
Glycosphingolipid catabolic process	11	5	>5	+	7.42E-03
Glycolipid catabolic process	12	5	>5	+	1.13E-02
Ceramide catabolic process	14	5	>5	+	2.39E-02
Phagocytosis, engulfment.	22	7	>5	+	5.44E-04
Sphingolipid catabolic process	20	6	>5	+	6.64E-03
Membrane lipid catabolic process	22	6	>5	+	1.15E-02
Glycosphingolipid metabolic process	32	8	>5	+	3.96E-04

Table 3.2. Gene ontology analysis of highly enriched genes from sorted MHCII^{hi} CD11c^{hi} : CD103⁺CD11b⁻, CD103⁺CD11b⁺ and CD103⁻CD11b⁺ cell subsets isolated from steady-state colons.

CD103⁺CD11b⁻ DCs (Day 4 DSS)

Gene ontology (Biological process)	Total number of genes in database	Number of gene hits	PANTHER over-representation test score	+/-	P-value
Regulation of B cell differentiation	25	6	>5	+	2.15E-02
Regulation of B cell	60	8	>5	+	3.66E-02

CD103⁺CD11b⁺ DCs (Day 4 DSS)					
Gene ontology (Biological process)	Total number of genes in database	Number of gene hits	PANTHER over-representation test score	+/-	P-value
Fc-gamma receptor signaling pathway	7	4	>5	+	2.95E-02
Fc receptor signaling pathway	13	5	>5	+	1.21E-02
Positive regulation myeloid leukocyte mediated immunity	27	8	>5	+	6.53E-05
Regulation of myeloid leukocyte mediated immunity	44	10	>5	+	1.15E-05
Regulation of mast cell activation	28	6	>5	+	3.10E-02
Regulation of mast cell degranulation	28	6	>5	+	3.10E-02
PRR signaling pathway	57	11	>5	+	9.08E-06
Toll-like receptor signaling pathway	37	7	>5	+	1.10E-02
Regulation of mast cell activation	38	7	>5	+	1.31E-02
CD103⁺CD11b⁺ Macrophages (Day 4 DSS)					
Gene ontology (Biological process)	Total number of genes in database	Number of gene hits	PANTHER over-representation test score	+/-	P-value
Fc-gamma receptor signaling pathway	7	4	>5	+	2.95E-02
Fc receptor signaling pathway	13	5	>5	+	1.21E-02
Positive regulation myeloid leukocyte mediated immunity	27	8	>5	+	6.53E-05
Regulation of myeloid leukocyte mediated immunity	44	10	>5	+	1.11E-05
Regulation of mast cell activation	28	6	>5	+	3.10E-02
Regulation of mast cell degranulation	28	6	>5	+	3.10E-02
PRR signaling pathway	57	11	>5	+	9.08E-06
Toll-like receptor signaling pathway	37	7	>5	+	1.10E-02
Regulation of mast cell activation	38	7	>5	+	1.31E-02

Table 3.3. Gene ontology analysis of highly enriched genes from sorted MHCII^{hi} CD11c^{hi} : CD103⁺CD11b⁻, CD103⁺CD11b⁺ and CD103⁻CD11b⁺ cell subsets isolated from day 4 DSS-treated colons.

Gene ontology analysis of highly expressing genes from steady-state as well as day 4 DSS treated sorted CD103⁺CD11b⁻ DC subset suggests the importance of this subset in regulating B cell activity. In steady-state, CD103⁺CD11b⁻ DCs are involved in regulating B cell activation and differentiation in the colonic LP (Table 3.2). Other hits include the metabolism of membrane fatty-acid components suggesting the possible cytoplasmic catabolism of membrane endocytic vesicles arising from high phagocytic activities (Table 3.2). In acute inflammatory settings (day 4 DSS), CD103⁺CD11b⁻ DCs are suggested to be involved in B cell differentiation and proliferation (Table 3.3). This subset may also be involved in positive regulation of TNF superfamily cytokine production as well as leukocyte chemotaxis and migration, in steady state and acute inflammation. CD103⁺CD11b⁺ DCs on the other hand exhibit upregulation in Fcγ-receptor signaling pathway suggesting this DC subset involvement in opsonisation and phagocytosis of antigens in steady-state and acute inflammation. Similar to CD103⁺CD11b⁻ DCs, CD103⁺CD11b⁺ DCs also show upregulation of gene expression involved in metabolism of membrane fatty-acid components. However, this function is strongly associated in steady state environment (Table 3.2). Analysis of genes upregulated in acute inflammatory environment suggests CD103⁺CD11b⁺ DCs acquire pro-inflammatory functions such as activation of PRR and TLR signaling pathways, regulation of myeloid leukocyte immunity, and regulation of mast cell activation and degranulation (Table 3.2). The third CD103⁻CD11b⁺ macrophage subset, seems to share a fair functional overlap with CD103⁺CD11b⁺ DCs. In steady state, CD103⁻CD11b⁺ macrophages upregulate genes associated with high phagocytic activity, suggesting their possible role in sampling of antigens within their local milieu (Table 3.2). In acute inflammatory environment, CD103⁻CD11b⁺ macrophages acquire pro-inflammatory functions similarly observed in CD103⁺CD11b⁺ DCs (Table 3.3).

3.1.3 Mutually-exclusive expression of Clec9A (DNGR1) and Clec4a4 (DCRI2) lectins on colonic CD103⁺ bona-fide DC subsets.

From our microarray analysis, the expression of two C-type lectins; Clec9A (DNGR1) and Clec4a4 (DCRI2) have been found to be respectively expressed on CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs. Scrutinizing these two lectins as potential specific markers for identification of DC subsets, we next validated their expression on all subsets using flow-cytometry. Colonic leukocytes were isolated as described in materials and methods section. CD45⁺ cells were gated to reveal CD11c^{hi}MHCII⁺ and CD11c^{int}MHCII⁺. As previously done, CD11c^{hi}MHCII⁺ cells were gated to analyze CD103⁺CD11b⁻, CD103⁺CD11b⁺ and CD103⁻CD11b⁺ subsets.

Figure 3.3.Flow-cytometric analysis of colonic CD11c⁺MHCII⁺ myeloid cells for expression of Clec9A and Clec4a4. Clec9A is expressed in CD103⁺CD11b⁻ DCs while Clec4a4 expression is restricted to CD103⁺CD11b⁺ DCs. Isolated colon leukocytes were stained with anti-MHCII, CD11c, CD11b, CD103, Clec9A and Clec4a4 monoclonal antibodies. Histograms show expression of Clec4a4 and Clec9A on various CD11c⁺MHCII⁺ subsets. Shaded grey peaks represent antibody isotype controls for anti- DCRI2 and anti- Clec9A.

Clec9A was found to be expressed in only CD103⁺CD11b⁻ DCs while Clec4a4 was expressed specifically in CD103⁺CD11b⁺ DCs. Expression of both lectins were not detected in CD103⁻CD11b⁺ macrophage and also in the CD11c^{int}MHCII⁺ macrophages(Figure 3.3).

The specific expression of Clec9A and Clec4a4 respectively in CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs, suggests their possible application in not only to identify the two DC subsets, but also in specific *in vivo* conditional ablation strategies which will be demonstrated in section 3.2.

3.1.4 Clec4a4⁺ CD103⁺CD11b⁺ DCs, Clec9A⁺ CD103⁺CD11b⁻ DCs and CX3CR1⁺ macrophages are localised within different anatomical sites in mouse colon.

We demonstrated both in the microarray and FACs analysis, Clec9A and Clec4a4 expression was specific to CD103⁺CD11b⁻ and CD103⁺CD11b⁺ colonic bona fide cDCs respectively. Expression of these two lectins was not detected in other CD11c⁺MHC⁺ subsets. Hence these two lectins proved to be useful specific markers for identifying these bona fide DC subsets in mouse colon. Next, we analyzed the anatomical locations of the two DC subsets in acetone-fixed distal colon cryosections. To study the anatomical locations of the two DC subsets in steady state as well as in early inflammation, distal colons were collected from control and DSS-treated (day 4), 8 weeks old female BALB/c mice. 8µm cryosections were stained with monoclonal antibodies (anti -Clec9a,-Clec4a4 and -CD11c). We included colons of CX3CR1-GFP mice to visualize CX3CR1⁺ macrophages and monocytes. As shown in figure 3.4A, Clec9A⁺ CD103⁺ CD11b⁻ DCs, representing the major cDC subset of the colon, are found scattered in the colonic LP and localized to a basal region in colonic innate lymphoid follicles (ILFs). In the lamina propria, Clec9A⁺ DCs can be found in close proximity with the epithelium as well and within the deep lamina propria (Figure 3.4C). Interestingly, Clec4a4⁺CD103⁺CD11b⁺ DCs were not detectable in the lamina propria but were localized abundantly, at the apical regions of ILFs (Figure 3.4A). CX3CR1-GFP⁺ macrophages were also detected in the ILFs (Figure 3.4B). Clec4a4⁺ DCs could not be detected in lamina propria even after DSS-treatment (Figure 3.4C). However, an increase of CX3CR1-GFP⁺ cells in lamina propria, presumably inflammatory monocytes, was observed in DSS-treated colons (Figure 3.5D). On

closer analysis of the CX3CR1-GFP⁺ population using flow-cytometry, the increase influx of CX3CR1-GFP⁺ cells observed in the sections of DSS-treated colons was indeed the CD64^{int}CX3CR1^{int} subset, which has been described as inflammatory monocytes (Figure 3.4E and 3.4F) [288]. In contrast, the CD64^{hi}CX3CR1^{hi} population, representing steady-state resident macrophages [287], was found to be significantly reduced in the DSS treated group (Figure 3.4E and 3.4F). Taken together our confocal analysis revealed Clec9A DCs and CX3CR1⁺ macrophages/monocytes were localized in ILFs and lamina propria, while Clec4a4⁺ DCs were restricted to ILFs, in both steady state and acute inflammatory conditions.

Figure 3.4. Confocal analysis illustrating differential localization of DC subsets and CX3CR1⁺ macrophages of steady state and acute DSS-inflamed distal colons. (A) Confocal image analysis of Clec9A⁺ (Red) and Clec4a4⁺ (Blue) DCs are localised in ILFs of steady state distal colons. (B) CX3CR1-GFP⁺ macrophages in ILF of steady state distal colon (Left). Hematoxylin and eosin staining of distal colon comprising of an ILF and LP (Right). (C) Confocal image analysis of Clec9A⁺ (Red) and Clec4a4⁺ (Blue) DCs in lamina propria of steady state and after day 4 DSS treatment. Clec4a4⁺ DCs were not found in lamina propria even after DSS treatment. (D) CX3CR1-GFP⁺ macrophages in lamina propria of steady state

and after day 4 DSS treatment. An increase influx of CX3CR1⁺ cells were observed in lamina propria after day 4 DSS treatment. (E) FACS analysis of colon LP CX3CR1⁺ cells isolated from steady state and day 4 DSS treatment. Numbers on FACS plots reflect the percentages of cells from the gated population. (F) Percentages of various CX3CR1⁺ populations in steady state and DSS treated colons (day 4, n=5). Sections from (A and C) were co-stained with PE-labeled anti-Clec9A, APC-labeled anti-Clec4a4 and FITC-labeled anti-CD11c. Sections B and D were co-stained with DAPI (Blue). All sections acquired at 20x magnification. Scale bars 50µm.

3.2 Conditional ablation of CD103⁺ dendritic subsets in DTR transgenic mice.

We have demonstrated Clec9A and Clec4a4 expression to be specific to CD103⁺CD11b⁻ and CD103⁺CD11b⁺ cDCs respectively. Here, we explore the potential of using both markers to specifically ablate CD103⁺CD11b⁻ and CD103⁺CD11b⁺ cDCs subsets in a transgenic DTR mouse system.

3.2.1 Efficient ablation of Clec9A⁺CD103⁺CD11b⁻ gut DC subset in Clec9A DTR mice.

Clec9A-DTR transgenic mice have been generated by our lab and has been used previously to study the consequence of depleting spleen CD8⁺CD11b⁻ DCs in a model of cerebral malaria [292]. To test the efficiency of depleting CD103⁺CD11b⁻ DCs in the colon and mesenteric lymph nodes, CX3CR1^{GFP}/Clec9A-DTR mice were used. Mice were injected i.p with 20ng/g body-weight of diphtheria toxin (DT) at -24h and -48h, and subsequently sacrificed. Colons and mesenteric lymph nodes were dissected and processed to isolate CD45⁺ leukocytes which were then stained for CD45, MHCII, CD11c, CD103, CD11b, CD8 and CD64. FACs analysis of isolated leukocytes were done as previously demonstrated (Section 3.1.1)

Figure 3.5. FACs analysis illustrating specific depletion of CD103⁺CD11b⁻ cDCs in Clec9A-DTR. (A) Administration of DT schedule. CX3CR1^{GFP}/Clec9A-DTR mice were i.p injected with 20ng/g bwt DT given at -48h and -24h. Colons and MLNs were subsequently removed for isolation of CD45⁺ leukocytes. Representative FAC plots depicting specific and efficient depletion of (B) CD11c^{hi}MHCII⁺ CD103⁺CD11b⁻ DC subset in colon ($n=3$) and (C) Optiprep gradient-purified MLN cells from 3 pooled mice consisting of CD11c^{hi}MHCII^{hi}CD103⁺CD11b⁻ migratory DCs and CD11c^{hi}MHCII^{int}CD8⁺CD11b⁻ lymphoid resident DCs in mesenteric lymph nodes. (D) Percentages and absolute numbers of gated cell populations in colonic LP ($n=3$) and pooled MLN ($n=3$). Numbers on FACs plots reflect the percentages of cells from the gated population. All statistical analysis were done using student's t -test (** $p < 0.01$).

As demonstrated in figure 3.5B, CD103⁺CD11b⁻ colonic DCs were efficiently ablated in Clec9A-DTR after single dose of DT. Other subsets in the CD11c^{hi}MHCII⁺ (gate 1), as well as CX3CR1⁺ macrophages/monocytes in the CD11c^{int}MHCII⁺ (gate 2) were not affected. In figure 3.5C, MLN migratory MHCII^{hi}CD103⁺CD11b⁻ and lymphoid resident MHCII^{int}CD8⁺CD11b⁻ DCs were also efficiently ablated in Clec9A-DTR mice. This proved the effectiveness of our Clec9A-DTR mice to ablate CD103⁺CD11b⁻ colonic as well as CD103⁺CD11b⁻ and CD8⁺CD11b⁻ MLN DCs.

3.2.1.1 Prolonged ablation of colonic Clec9A⁺CD103⁺CD11b⁻ DC subset does not result in loss of other CD11c^{hi}MHCII^{hi} subsets or exhibit DT mediated toxicity in Clec9A-DTR.

Since Clec9A is also expressed in common dendritic cell progenitors (CDPs) and pre-DCs in the bone-marrow [293], it is possible these cell fractions would be depleted. Depletion of these DC progenitors would impact the numbers of the other CD103⁺CD11b⁺ DC subset. In addition, mice receiving repeated doses of DT administered over long periods may not only show toxicity, but also develop neutralizing antibodies which may affect the effectiveness of this ablation system. To rule out this possibility, we injected WT and Clec9A-DTR mice with repeated doses of DT (20ng/g bwt) every 72h for 15 days as illustrated in figure 3.6A. Body weights were monitored daily to observe for weight loss mediated by DT toxicity. WT and Clec9A-DTR colons were excised at day 16 DT-treatment, to analyze CD11c^{hi}MHCII⁺ subsets.

WT and Clec9A-DTR mice did not exhibit signs of distress nor do they succumbed to repeated exposure to DT. Both mouse groups maintained their body weights over the course of repeated DT injection (Figure 3.6B). Most importantly, repeated exposure to DT in Clec9A-DTR did not affect the CD103⁺CD11b⁺ DC subset and CD103⁻CD11b⁺ macrophages even till day 16. In addition, Clec9A-DTR mice did not develop resistance to DT since the target CD103⁺CD11b⁻ DC subset was still efficiently depleted at day 16 post DT treatment (3.6C).

Figure 3.6. Observation of Clec9A-DTR mice subjected to prolonged DT treatment. (A) Administration schedule of i.p 20ng/g bwt DT in a span of 16 days. (B) Body weight monitoring of WT and Clec9A-DTR mice during 15 days DT treatment described in A. No body weight loss associated with DT induced toxicity observed in both WT and Clec9A-DTR mice. (C) FACS plots depicting efficient depletion of CD103⁺CD11b⁻ DCs at day 16 without affecting other CD11c⁺MHCII^{hi} subsets. Numbers on FACS plots reflect the percentages of cells from the gated population. (D) Percentages and absolute numbers of gated CD11c⁺MHCII^{hi} cell populations in colonic LP ($n=3$) of WT vs Clec9A DTR at day 16 DT treatment. All statistical analysis were done using student's t -test (** $p < 0.01$).

3.2.1.2 Partial ablation of CD11c^{int}SiglecH⁺gut plasmacytoid DC subset in Clec9A DTR mice.

We have demonstrated the efficiency of Clec9A-DTR in depleting colonic CD103⁺CD11b⁻ DCs as well MLN migratory CD103⁺CD11b⁻ and lymphoid resident CD8⁺CD11b⁻ DCs. However, Clec9A has also been shown to be expressed by Siglec-H⁺ plasmacytoid dendritic cells [294]. In an attempt to validate depletion of gut pDC population, Clec9A-DTR as well as Siglec-H-DTR were injected i.p 20ng/g bwt DT. MLNs were isolated and after 24h DT treatment and processed to obtain single cell suspension as described in materials and method.

Figure 3.7. FACS analysis of MLN for depletion of pDCs. Siglec-H-DTR effectively ablates CD11c^{int}Siglec-H⁺ pDCs while Clec9A-DTR shows partial ablation. Numbers on FACS plots represent a percentage of total MLN cells.

From our analysis, CD11c^{int}Siglec-H⁺ pDCs were completely ablated in Siglec-H-DTR while Clec9A-DTR showed only partial ablation (Figure 3.7). We were currently unable to isolate colonic Siglec-H⁺ pDCs hence we were unable to demonstrate depletion of pDCs in Clec9A-DTR colon. However we speculate colonic pDCs to be partially ablated in Clec9A-DTR hence Siglec-H-DTR will be used as a control in interpretation of results in further sections.

3.2.2 Ablation of Clec4a4⁺CD103⁺CD11b⁺ gut DC subset in Clec4a4 DTR mice.

The specific expression of Clec4a4 on CD103⁺CD11b⁺ DCs as demonstrated in our microarray (Figure 3.2) and FACs analysis (Figure 3.3) has enabled us to generate DTR mice capable of depleting CD103⁺CD11b⁺ DCs. Generation of Clec4a4-DTR has been described in materials and method section. To analyze the efficiency of our Clec4a4-DTR mice in CD103⁺CD11b⁺ DC subset ablation, CX3CR1-GFP/Clec4a4-DTR mice were used. CX3CR1-GFP/Clec4a4-DTR mice were injected with 20ng/g bwt DT at -48h and -24h. MLNs and colons were dissected thereafter and processed to analyze depletion of CD103⁺CD11b⁺ DC subset.

Figure 3.8. FACs analysis illustrating specific depletion of CD103⁺CD11b⁺ cDCs in Clec4a4-DTR. CX3CR1^{GFP}/Clec4a4-DTR mice were i.p injected with 20ng/g bwt DT given at -24h. Colons and MLNs were subsequently removed for isolation of CD45⁺

leukocytes. FAC plots depicting specific and efficient depletion of (A) $CD11c^{hi}MHCII^{+}CD103^{+}CD11b^{+}$ DC subset in colon and (B) $CD11c^{hi}MHCII^{hi}CD103^{+}CD11b^{+}$ migratory and $CD11c^{hi}MHCII^{int}CD8^{-}CD11b^{+}$ lymphoid resident DCs in mesenteric lymph nodes. The $CD11c^{int}MHCII^{+}CX3CR1-GFP^{hi}$ macrophages were also depleted in our Clec4a4-DTR (gate 2, lower panel). (C) Percentages and absolute numbers of gated cell populations in colonic LP ($n=3$) and pooled MLN ($n=3$). FACs plots of colonic LP cells were representative of three different mice. FACs plots of MLN cells were obtained from a pool of 3 mice enriched by an optiprep gradient. Numbers on FACs plots reflect the percentages of cells from the gated population. All statistical analysis were done using student's *t*-test (* $p < 0.05$, *** $p < 0.001$).

As demonstrated in figure 3.8, $CD11c^{hi}MHCII^{+}CD103^{+}CD11b^{+}$ DC subset is ablated by 70% in colon and 50% in MLNs after single dose of DT, while MLN $CD11c^{int}MHCII^{+}CD8^{-}CD11b^{+}$ resident lymphoid DCs were depleted by 80%. However, for some unknown reasons, $CD11c^{int}MHCII^{+}CX3CR1-GFP^{hi}$ macrophages were depleted in our Clec4a4-DTR (Figure 3.8A, gate 2, lower right panel). This non-specific ablation of $CX3CR1-GFP^{hi}$ macrophages may affect the interpretation of results generated from the use of Clec4a4-DTR.

Clec4a4-DTR ablates the $CD11c^{int}MHCII^{+}CX3CR1-GFP^{hi}$ macrophages similarly observed in our CD169-DTR. Here, we show the ablation of $CX3CR1-GFP^{hi}$ steady state macrophages in our CD169-DTR (Figure 3.9). Hence CD169-DTR will be used as a control in interpretation of results involving the use of Clec4a4-DTR in further sections.

Figure 3.9. FACS analysis of colon leukocytes isolated from CD169-DTR. (A) Specific CD11c^{int}MHCII^{hi}CX3CR1-GFP^{hi} macrophage depletion in CD169-DTR colon as observed in our Clec4a4 DTR after -48 and -24h 20ng/g bwt DT treatment. No depletion was observed in other CD11c⁺MHCII⁺ subsets. (B) Percentages and absolute numbers of gated cell populations in colonic LP ($n=3$) of WT vs CD169-DTR after DT injections. Numbers on FACS plots reflect the percentages of cells from the gated population. All statistical analysis were done using student's t -test (***) $p < 0.001$.

3.2.3 Ablation of either Clec4a4⁺CD103⁺CD11b⁺ or Clec9A⁺CD103⁺CD11b⁻ DC subset does not affect anatomical location of the other subset.

We have demonstrated in figure 3.4, Clec9A⁺CD103⁺CD11b⁻ DCs were scattered in LP and localized in colonic ILFs while Clec4a4⁺CD103⁺CD11b⁺ DCs were localized only in colonic ILFs. However, we have not ruled out the possibility that depletion of either one DC subset could result in functional compensation by the other DC subset, hence leading to underestimation of the physiological role of the depleted subset. We raise the question if this compensation of function would lead to the migration of the unablated DC subset to the anatomical site of the ablated DC subset. Hence, we next evaluated using confocal fluorescence microscopy if ablation of either Clec4a4⁺CD103⁺CD11b⁺ or Clec9A⁺CD103⁺CD11b⁻ DC subset would have an effect on the anatomical location of the other subset. To achieve this, we obtained distal colon sections from day 4 DSS-treated Clec9A- and Clec4a4-DTR mice. Colons were processed for cryo-sectioning as described in materials and method. To visualize DC subsets, colon sections were co-stained with FITC-conjugated CD11c, PE-conjugated Clec9A and APC-conjugated Clec4a4 monoclonal antibodies.

Our confocal analysis did not reveal any transmigration of DC subset to the anatomical location of the depleted DC subset during early inflammation (Figure 3.10). Most importantly, it is clear that Clec4a4⁺CD103⁺CD11b⁺ DCs were still localized to ILFs and were never found in LP after depletion of Clec9A⁺CD103⁺CD11b⁻ DCs during acute inflammation.

Day 4 DSS

Figure 3.10. Confocal analysis of colonic ILFs and LP after depletion of Clec9A⁺ and Clec4a4⁺ DCs. Cryofrozen distal colons were obtained from Clec9A-DTR and Clec4a4-DTR at day 4 DSS treatment to resemble early inflammation. Colon slices were co-stained with FITC-conjugated CD11c, PE-conjugated Clec9A and APC-conjugated Clec4a4 monoclonal antibodies. Confocal fluorescence microscopy was used to analyze anatomic locations of Clec9A⁺ and Clec4a4⁺ DCs in ILFs (entire left panel) and LP (entire right panel) as a consequence of ablating either subset during early inflammation. Ablation of either subset did not affect the anatomical locations of the unablated subset. Hematoxylin and eosin staining of distal colon comprising of ILF and LP of day 4 DSS treated Clec9A-DTR and Clec4a4-DTR colon (lowest panel). (20X magnification, scale bars 50µm).

3.2.4 Conclusion

To study the functions of DC subsets *in vivo*, our lab generated two DTR mice capable of depleting CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DC subsets. We demonstrated Clec9A-DTR mice capable of efficiently depleting CD103⁺CD11b⁻ DC in colon after a single dose of DT. Ablation of CD103⁺CD11b⁻ DCs in Clec9A-DTR was still efficient at 15 days repeated exposure to DT without affecting other MHCII⁺CD11c⁺ subsets. In addition, Clec9A-DTR exhibits no signs of DT-mediated toxicity or distress during the entire 15 day course of DT treatment. Clec4a4-DTR have been generated in our lab to specifically ablate CD103⁺CD11b⁺ DCs. Here, we demonstrated after single dose of DT, CD103⁺CD11b⁺ DCs in colon were efficiently ablated. Similar to the Clec9A-DTR, Clec4a4-DTR mice exhibits no signs of distress or DT-mediated toxicity after repeated exposures to DT over a 15 day period (data not shown). Finally, depletion of either DC subset does not result in any movement of DC to the anatomical location of the depleted DC subset during early inflammation. Most importantly, it is clear that Clec4a4⁺CD103⁺CD11b⁺ DCs were still localized to ILFs and were never found in LP after depletion of Clec9A⁺CD103⁺CD11b⁻ DCs during acute inflammation.

Clec9A-DTR have been observed to partially ablate MLN CD11c^{int}Siglec-H⁺ pDCs and possibly too in the colon, while Clec4a4-DTR, for unknown reasons depletes colonic CD11c^{int}MHCII^{hi}CX3CR1-GFP^{hi} subset of macrophages. Therefore Siglec-H-DTR (depleting CD11c^{int}Siglec-H⁺ pDCs) and CD169-DTR (depleting CD11c^{int}MHCII^{hi}CX3CR1-GFP^{hi} macrophages) were included in our experiment internal controls as a comparison to Clec9A-DTR and Clec4a4-DTR respectively.

3.3 Consequence of depleting CD103⁺ dendritic cell subsets on severity of DSS-induced colitis.

A network of antigen-presenting immune cell subsets residing in the mucosal lamina propria and below the intestinal epithelium, plays an important role in balancing between protective immune responses and tolerance to various harmless food antigens, commensals and pathogens. It has been shown that in CD11c-DTR, depletion of total CD11c⁺ APCs before DSS treatment, ameliorated DSS induced colitis [295]. This finding suggests CD11c⁺ APCs have a pro-inflammatory function. However, we and others have demonstrated that colonic CD11c⁺MHCII⁺ APCs consists of multiple cell lineages and subsets (section 3.1) each with their own specialized functions. Hence their relative contributions to inflammation still remains unclear.

Therefore, in this section, we shall demonstrate the consequence of depleting CD11c^{hi}MHCII⁺CD103⁺CD11b⁻ and CD11c^{hi}MHCII⁺CD103⁺CD11b⁺ colonic DC subsets using Clec9A- and Clec4a4-DTR respectively, to elucidate their contributions to inflammation in a DSS-induced colitis model.

3.3.1 Depletion of Clec9A⁺CD103⁺CD11b⁻ DCs but not Clec4a4⁺CD103⁺CD11b⁺ DCs exacerbates DSS-induced colitis.

We aim to elucidate the function of Clec9A⁺CD103⁺CD11b⁻ and Clec4a4⁺CD103⁺CD11b⁺ DCs in regulating colonic inflammation by analyzing the consequence of ablating each subset in a mouse model of DSS induced colitis. DSS concentration of 2% supplied in drinking water was fed to 8 week old female WT, Clec9A- and Clec4a4-DTR water for 7 days to induce mild colitis. Mice were given fresh drinking water thereafter for another 8 days. To maintain the ablation of colonic DC subsets throughout the DSS regiment, Clec9A- and Clec4a4-DTR were injected DT every 3 days as illustrated in fig 3.11A. Mice were observed for clinical signs of colitis which includes rapid body-weight loss, presence of fecal occult blood, colon shrinkage and loss of colonic epithelial barrier integrity.

From our data, 2% DSS only resulted in mild colitis in WT mice. However, under the same DSS regiment, Clec9A-DTR suffered exacerbated colitis marked by severe weight loss and colon shrinkage, highest colonic epithelial damage leading to increased FITC-dextran in serum, and increased fecal blood scores as compared to

Figure 3.11. Ablation of $Clec9A^{+}CD103^{+}CD11b^{-}$ DCs but not $Clec4a4^{+}CD103^{+}CD11b^{+}$ DCs exacerbates DSS-induced colitis. (A) WT, Clec4a4- and Clec9A-DTR mice were given 2% DSS in drinking water for 7 days and water 8 days thereafter. DTR mice were given 20ng/g bwt DT as illustrated. (B) Body weight measurements expressed as % of initial weight, of WT, Clec9A- and Clec4a4-DTR subjected to a 2% DSS regimen described in A (n=8). (C) Colon lengths in cm of WT, Clec9A- and Clec4a4-DTR measured at day 10 DSS regimen (n=8). (D) Assessment of intestinal permeability of DSS-treated WT, Clec4a4- and Clec9A-DTR using FITC-conjugated dextran as an epithelial permeability probe, administered through oral gavage. Concentration of FITC-dextran was measured from serum. Assay was carried out on day 4 and 10 DSS treatment (n > 7). (E) Faecal occult blood scores of WT, Clec9A- and Clec4a4-DTR faecal samples collected at day 8 DSS treatment (n > 9). All statistical analysis were done using student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

WT and Clec4a4-DTR (Figure 3.11B-E). Clec9A-DTR also showed earlier onset of colitis than other groups. Interestingly, Clec4a4-DTR showed the least weight loss, hardly any colon shrinkage, and mild fecal occult blood scores when compared to WT. Colonic epithelium of Clec4a4-DTR mice seems to be the most intact of all groups as indicated by nearly undetectable levels of serum FITC-dextran at day 10 DSS treatment. In addition, Clec4a4-DTR mild weight loss occurs only at a much later time-point (day 9) (Figure 3.11B-E). Our data strongly suggest depletion of Clec9A⁺CD103⁺CD11b⁻ colonic DCs resulted in exacerbation of colitis while depletion of Clec4a4⁺CD103⁺CD11b⁺ DCs resulted in resistance. This observation is also consistent when Clec4a4-DTR were subjected to stronger DSS dose of 5%. When given a 5% DSS treatment, Clec4a4-DTR mice still exhibited resistance to colitis as indicated by mild weight loss over the entire period of DSS regiment (figure 3.11a). Clec9A-DTR mice when subjected to 5% DSS treatment, showed much severe colitis symptoms compared to WT. In fact, Clec9A-DTR mice showed dramatic weight loss after day 9 DSS treatment (>30%), and had to be euthanized as mandated by IACUC guidelines.

Figure 3.11a. Body-weight loss of WT and Clec4a4-DTR treated with 5% DSS. WT, Clec4a4- and Clec9A-DTR mice were given 5% DSS in drinking water for 7 days and fresh water 8 days thereafter. Daily body-weights were recorded over the course of DSS-regiment and expressed as % of initial weight. Statistical analysis was done using student's *t*-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 5$ mice per group).

Next, we evaluated H.E stained distal colons from day 10 2% DSS-treated WT, Clec9A- and Clec4a4-DTR (Figure 3.12). Consistent with the above observations, DSS-treated Clec9A-DTR exhibited the highest degree of epithelial erosion and mucosal damage as compared to WT (Figure 3.12C). In fact, the entire colonic architecture including colonic crypts and lamina propria was completely disrupted in Clec9A-DTR while WT distal colons only showed moderate epithelial erosion and mucosal damage (Figure 3.12B). In addition, presence of inflammatory cells infiltrating the inflamed colon localized at the sub-mucosal and mucosal regions were highest in Clec9A-DTR (Figure 3.12C). The resistance of Clec4a4-DTR to DSS-induced colitis was also reflected in H.E stained distal colons. H.E stained sections of Clec4a4-DTR distal colons showed only very mild and almost negligible epithelial erosions and mucosal damage (Figure 3.12D). Overall, Clec4a4-DTR distal colons exhibited least presence of inflammatory infiltrates in the submucosal and mucosal regions indicating the mild extent of colonic inflammation (Figure 3.12D).

Since we have demonstrated Clec9A-DTR also partially ablates Siglec-H⁺ pDCs (Figure 3.7), we rule out the possibility of this ablation having a consequence of exacerbating DSS-induced colitis. To achieve this, Siglec-H-DTR mice were used as an internal control alongside WT and Clec9A-DTR in our DSS-induced colitis regiment. WT, Siglec-H- as and Clec9A-DTR were subjected to DSS treatment as illustrated in figure 3.13.

Figure 3.12. Ablation of $\text{Clec9A}^+\text{CD103}^+\text{CD11b}^-$ DCs but not $\text{Clec4a4}^+\text{CD103}^+\text{CD11b}^+$ DCs exacerbates DSS-induced colitis. H.E stained distal colons isolated from (A) Steady-state as well as day 10 DSS treated (B) WT, (C) Clec9A- and (D) Clec4a4-DTR. Sections were displayed as representative images from 2 individual mice per group. (Brightfield images, 20x magnification. Scale bars 50 μm).

Here, we report that depletion of pDCs in Siglec-H-DTR had no observable consequence in our DSS-induced colitis model. Siglec-H-DTR did not exhibit rapid and severe body-weight loss as observed in Clec9A-DTR. In fact, Siglec-H-DTR mice behaved similarly to WT mice subjected to similar DSS regimen (Figure 3.13).

Hence we rule out the potential contribution of non-specific pDC depletion in the exacerbated colitis observed in Clec9A-DTR.

Figure 3.13. Weight loss of DSS-treated WT, Siglec-H-DTR subjected to 2% DSS induced colitis regiment. Ablation of SiglecH⁺ pDCs did not exacerbate DSS-induced colitis. Weights are expressed as % of initial bodyweight. Statistical analysis was done using student's *t*-test (* $p < 0.05$, ** $p < 0.01$, $n = 3-5$ mice per group).

For the Clec4a4-DTR mice, we observed a non-specific partial ablation of CD11c^{hi}MHCII⁺CX3CR1-GFP^{hi} colonic resident macrophages after DT treatment (Figure 3.8A lower right). To rule out the possibility that non-specific depletion of this macrophage population contributes to colitis resistance observed in Clec4a4-DTR, CD169-DTR capable of specifically depleting CD11c^{hi}MHCII⁺CX3CR1-GFP^{hi} colonic resident macrophages (Figure 3.9) were used as internal control. Depletion of CD11c^{hi}MHCII⁺CX3CR1-GFP^{hi} colonic macrophages in CD169-DTR did not result in resistance to colitis as observed in Clec4a4-DTR. On the contrary, CD169-DTR showed rapid and severe body weight loss as compared to WT mice (Figure 3.14A). Similar to Clec9A-DTR, CD169-DTR demonstrated other symptoms of increased susceptibility to DSS-induced colitis which includes significant colon shrinkage (Figure 3.14B), increased presence of fecal occult blood (Figure 3.14C) and loss of colonic epithelial permeability (Figure 3.14D). Hence we report that the colitis resistance observed in Clec4a4-DTR mice is contributed by depletion of Clec4a4⁺CD103⁺CD11b⁻ DCs but not CD11c^{hi}MHCII⁺CX3CR1-GFP^{hi} colonic macrophages.

Figure 3.14. Ablation of colonic $CD11c^{int}MHCII^{+}CX3CR1-GFP^{hi}$ macrophages exacerbates DSS-induced colitis. WT and CD169-DTR mice were given 2% DSS in drinking water for 7 days and water 8 days thereafter. DTR mice were given 20ng/g bwt DT as illustrated (Figure 3.10A). (A) Body weight measurements expressed as % of initial weight, of WT and CD169-DTR subjected to a 2% DSS regiment described in A ($n=5$). (B) Colon lengths in cm of WT and CD169-DTR measured at day 10 DSS regiment ($n=5$). (C) Fecal occult blood scores of WT and CD169-DTR fecal samples collected at day 8 DSS treatment ($n > 7$). (D) Assessment of intestinal permeability of DSS-treated WT and CD169-DTR using FITC-conjugated dextran as an epithelial permeability probe, administered through oral gavage. Concentration of FITC-dextran was measured from serum. Assay was carried out on day 4 and 10 DSS treatment ($n=5-9$). All statistical analysis were done using student's *t*-test (Significant values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3.2 Depletion of $Clec9A^{+}CD103^{+}CD11b^{-}$ DC subset results in increased colonic infiltration of neutrophils and inflammatory monocytes during early-stage DSS-induced colitis.

Neutrophil and monocyte accumulation in inflamed colons has been identified as a hallmark of colitis where the severity of the disease is correlated with the increased trafficking of both inflammatory cells in the mucosa [296]. Using FACS analysis, we next analyzed the population of $CD11b^{+}Ly6G^{+}$ neutrophils and $CD11b^{+}Ly6C^{+}$

Figure 3.15. Analysis of inflammatory cells in colon at day 4 DSS treatment. WT, Clec4a4⁻ and Clec9A-DTR colons were analyzed for presence of inflammatory monocytes (CD11b⁺Ly6C⁺) and neutrophils (CD11b⁺Ly6G⁺) at day 4 DSS treatment. (A) FACS plots depicting gating strategy of CD11b⁺Ly6G⁺ neutrophils (upper panel) and CD11b⁺Ly6C⁺ monocytes (lower panel) in steady state and day 4 DSS-treated colons. Representative dot plots of 3 mice per group (B) Percentages of CD11b⁺Ly6G⁺ neutrophils and CD11b⁺Ly6C⁺ monocytes in steady state and day 4 DSS treated colons ($n=3-6$ mice). Percentages on FACS plots represent from total colonic CD45⁺ cells. All statistical analysis was done using student's *t*-test (Significance: ** $p < 0.01$).

monocytes in steady state as well as day 4 DSS-treated WT, Clec4a4⁻ and Clec9A-DTR mice. Consistent to what was observed in histological sections (3.12C), day 4 DSS-treated Clec9A-DTR colons show significantly higher influx of both CD11b⁺Ly6G⁺ neutrophils ($p < 0.01$) and CD11b⁺Ly6C⁺ monocytes ($p < 0.01$) (Figure 3.15). However, this observation was not observed in DSS treated Clec4a4-DTR where the colonic influx of both inflammatory cell types were not significantly different from DSS treated WT mice (Figure 3.15). These observations clearly points that depletion of CD103⁺CD11b⁻ colonic DCs in Clec9A-DTR but not

CD103⁺CD11b⁺ DCs in Clec4a4-DTR, resulted in increased susceptibility to DSS-induced colitis.

3.3.2.1 Exacerbation of DSS-induced colitis in Clec9A-DTR mice is not contributed by increased neutrophilia.

It has been observed in CD11c-DTR, depletion of CD11c⁺ DCs presents an undesirable side-effect known as neutrophilia [297]. This phenomenon has functional consequences as demonstrated in the above study using a mouse pyelonephritis model, the resultant increase in neutrophil numbers 24h after DC ablation led to more efficient bacterial clearance in the infected kidneys. However, infection before the 24h DC ablation period, before the onset of neutrophilia, led to impairment of bacterial clearance in infected kidneys. Depletion of CD11c⁺ DCs resulting in neutrophilia, possibly resulted from increased granulopoiesis in bone marrow.

Our Clec9A-DTR mice demonstrated symptoms of neutrophilia marked by increased colonic influx of neutrophils and monocytes at day 4 DSS treatment (Figure 3.15B). Hence we question if this has any functional consequence on the susceptibility of Clec9A-DTR to DSS-induced inflammation. Here, we show the increased susceptibility of Clec9A-DTR to DSS-induced colitis was a consequence of ablating CD103⁺CD11b⁺ DCs and not due to the increased numbers of Ly6G⁺ neutrophils as well as Ly6C⁺ monocytes infiltrating the colonic lamina propria (Figure 3.16). To achieve this, we depleted Ly6C⁺ monocytes and Ly6G⁺ neutrophils *in vivo* using 100µg anti-Gr1 depleting antibodies delivered i.p to WT and Clec9A-DTR during DSS treatment. The time-points of anti-Gr1 antibody injections and DSS regiment given to the experimental mice was summarized in figure 3.16A.

Figure 3.16. The exacerbation of DSS-induced colitis in Clec9A-DTR is not due to increased neutrophilia. (A) Schedule of DSS regimen over a span of 15 days. WT and Clec9A-DTR were fed 2% DSS supplied in drinking water *ad libitum* for 7 days and given fresh water thereafter for another 8 days. Injections of 20ng/g btw DT and 100μg anti-Gr1 depleting antibodies were administered as illustrated. (B) Body weight loss expressed as % initial weight of WT (open circles) and Clec9A-DTR (open squares) as well as anti-Gr1 treated WT (red circles) and Clec9A-DTR (red squares) (n=3). (C) Fecal occult blood scores of DSS-treated WT and Clec9A-DTR, with and without anti-Gr1 treatment (n=4). (D) Number of CD11b⁺Ly6C⁺ monocytes and CD11b⁺Ly6G⁺ neutrophils obtained from colons at day 16, 24hr after last injection of anti-Gr1. Monocytes and neutrophils were depleted by ~70% and ~50% respectively in both WT and Clec9A-DTR after administration of 100μg anti-Gr depleting antibodies. All statistical analysis were done using student's *t*-test (Significant values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Consistent with our earlier observations, non anti-GR1 treated Clec9A-DTR mice showed exacerbated DSS-induced colitis, marked by increased body-weight loss and fecal occult blood scores as compared to WT mice when subjected to similar DSS-regiment (Figure 3.16B-C). However, depletion of Ly6G⁺ neutrophils and Ly6C⁺ monocytes in WT but not Clec9A-DTR limits the severity of colitis as demonstrated by body weight loss and fecal occult blood scores (Figure 3.16B-C). Depletion of colonic Ly6G⁺ neutrophils and Ly6C⁺ monocytes using anti-Gr1 depleting antibodies was efficient with a reduction of ~75% in monocytes and ~50% neutrophils at day 16 after 24h administration of anti-Gr1 in both control and Clec9A-DTR. Taken together, our results clearly show the exacerbation of DSS-induced colitis in Clec9A-DTR is not due to increased neutrophilia, but specific ablation of CD103⁺CD11b⁻ DCs.

3.3.3 Depletion of Clec9A⁺CD103⁺CD11b⁻ DC subset results in increased numbers of colonic tertiary lymphoid tissues during DSS-induced inflammation.

It has been demonstrated in a study by Matthias *et al*, colonic tertiary lymphoid tissues can arise during inflammatory conditions to compensate for immunodeficiency, in their case, an absence of ROR γ t-mediated innate immunity [298]. DCs are important in maintaining immune homeostasis, by mediating tolerance to harmless antigens and launch protective inflammatory immune responses against pathogens. Hence we speculate in our DSS-induced colitis model, the depletion of CD103⁺CD11b⁻ DCs in Clec9A-DTR would lead to severe immune-deficiency resulting in increased formation of colonic tertiary lymphoid tissues as a compensatory mechanism, similarly observed in immune-compromised ROR γ t^{-/-} mice [298]. To test our hypothesis, we challenged 8 week old female WT and Clec9A-DTR mice with 2% DSS for 7 days, followed by fresh water for the next 10 days. Mice were sacrificed on day 10 for quantitation of colonic tertiary lymphoid tissues. To quantitate the number of colonic tertiary lymphoid tissues, cryofrozen 8 μ m tissue slices from swiss-rolled colons were immunostained with anti-B220 to reveal tertiary lymphoid tissues consisting of B cell follicles.

Figure 3.17. Formation of colonic tertiary lymphoid tissues during inflammation. (A) Fluorescence immuno-staining of a colonic tertiary lymphoid tissue (arrow). Colon cryosections were swiss-rolled and immuno-stained with FITC-conjugated anti-B220 monoclonal antibody. (40x magnification. Scale bar 25 μ m). (B) Dot plot charting the number of quantitated colonic tertiary lymphoid tissues from steady state WT, DSS-treated WT and DSS-treated Clec9A-DTR. A tissue slice was obtained at every 40 μ m intervals, total of 5 slices per swiss-rolled colon, from a total of 2 mice per group. All statistical analysis was done using student's *t*-test (Significance: ** $p < 0.01$, *** $p < 0.001$).

monoclonal antibody. Colonic lymphoid tissues were counted under fluorescence microscope.

Our data clearly shows depletion of CD103⁺CD11b⁻ DCs in colon resulted in significant increase in numbers of colonic tertiary lymphoid tissues ($p < 0.001$). This observation was also observed in ROR γ t-deficient mice [298] when subjected to similar DSS-treatment, as a compensatory mechanism to bolster a weakened immunity. Hence we propose the depletion of CD103⁺CD11b⁻ DCs in Clec9A-DTR results in weakened innate immunity resulting in increased formation of tertiary lymphoid tissues during DSS-induced injury, possibly to contain uncontrolled bacterial load.

3.3.4 Conclusion

In this chapter, we aim to elucidate the role of CD103⁺CD11b⁻ as well as CD103⁺CD11b⁺ colonic DCs in gut immunity. To achieve this Clec9A- and Clec4a4-DTR, capable of depleting CD103⁺CD11b⁻ and CD103⁺CD11b⁺ colonic DCs respectively, were used in a mouse model of DSS-induced colitis. From our data, it is clear that depletion of CD103⁺CD11b⁻ but not CD103⁺CD11b⁺ DCs resulted in exacerbation of colitis. Clec9A-DTR exhibits increased numbers of neutrophil and monocyte infiltration as early as at day 4 DSS treatment. We ruled out this effect of neutrophilia on the exacerbation of colitis in Clec9A-DTR because depletion of these inflammatory cells using anti-Gr1 antibodies failed to limit the severity of disease. In addition, Clec9A-DTR exhibits weakened gut immunity leading to excessive formation of tertiary lymphoid tissues, demonstrated as a compensation mechanism to cope with increased bacterial burden [298]. In contrast, depletion of CD103⁺CD11b⁺ DCs in Clec4a4-DTR resulted in increased resistance to colitis, high-lighting that these 2 subsets not only differ in terms of anatomical locations, but also in controlling intestinal inflammation. Taken together, our results highly suggest, CD103⁺ DC subsets oppositely regulates immune homeostasis; CD103⁺CD11b⁻ DCs regulate colonic inflammation while CD103⁺CD11b⁺ DCs have a pro-inflammatory role.

3.4 Elucidating mechanisms of CD103⁺ DC subsets in controlling colonic inflammation.

Intestinal dendritic cells are known to have a central role in balancing between protective and tolerogenic immune responses in the gut. By inducing tolerance to harmless antigens and protective immune responses to pathogens, dendritic cells are indispensable in ensuring exuberant immune responses resulting in inflammatory bowel diseases do not develop. We have demonstrated from previous section, CD103⁺CD11b⁻ DCs regulate colonic inflammation while CD103⁺CD11b⁺ DCs have a pro-inflammatory function. However the underlying mechanism of how these two DC subsets exert their opposing roles in controlling intestinal inflammation remains to be elucidated.

3.4.1 Depletion of Clec9A⁺CD103⁺CD11b⁻ or Clec4a4⁺CD103⁺CD11b⁺ DC subsets did not affect numbers and function of LP regulatory T cells, RORγt⁺ ILCs and γ/δ T17 cells at early stages of DSS-mediated colitis.

DCs have been long known to be professional antigen-presenting cells capable of initiating innate as well as adaptive immune responses. As a first step to unravel the mechanisms of how Clec9A⁺CD103⁺CD11b⁻ as well as Clec4a4⁺CD103⁺CD11b⁺ DCs exert their innate functions in controlling intestinal inflammation, we analyzed populations of LP immune cells such as CD4⁺Foxp3⁺ Tregs cells, SCART-2⁺γδ T17 cells as well as RORγt⁺ ILCs for the production of IL-22 cytokine after ablation of DC subsets. SCART-2 monoclonal antibodies have been recently used to mark a population of IL-17-secreting Vγ4⁺γδ⁺ T-cells [305-306].

IL-22, a cytokine critical for early defense against intestinal infections with *Citrobacter rodentium* [304], has been shown to be an important cytokine in maintaining epithelial integrity after DSS induced damage [299], and are expressed by innate immune cells such as NK cells and RORγt⁺ ILCs, as well as in T-cell lineages including αβ⁺ and γδ⁺ T cells [300]. To analyse these cell populations, RORγt^{GFP}/Clec9A-DTR and RORγt^{GFP}/Clec4a4-DTR were subjected to a four day DSS treatment. DT was administered to both DTR strains at day -1, 0 and 3 and mice were sacrificed at day 4 to analyze for CD4⁺Foxp3⁺ Tregs cells, SCART-2⁺γδ T17 cells as well as RORγt⁺ ILCs. Colon leukocyte suspensions purified from a 70/40%

percoll gradient were stimulated with PMA, ionomycin and brefeldine A as described in materials and methods. Cells were then immuno-labelled and analyzed by FACS.

Figure 3.18. Ablation of CD103⁺CD11b⁻ and CD103⁺CD11b⁺ colonic DCs did not affect numbers of CD4⁺Foxp3⁺ Treg as well as RORγt⁺ ILCs and SCART2⁺γδ T17 cells and their propensity to secrete IL-22. RORγt^{GFP}Clec9A⁻ and RORγt^{GFP}Clec4a4-DTR were injected with DT and subjected to 4 days DSS treatment to study the above cell populations, during early DSS induced inflammation. LP cells were isolated, immunolabeled and analyzed by FACS. (A) Percentage of LP CD4⁺Foxp3⁺ Treg cells. *n* > 8 mice per group, from 3 independent experiments. (B) Percentage of RORγt^{GFP} ILCs (upper panel) and IL-22-producing RORγt⁺ ILCs (lower panel), *n* = 3 to 6 mice per group, representative data of 2 independent experiments. (C) Percentage of γδ⁺ T-cells of total CD3⁺ population (upper panel), SCART2⁺γδ⁺ of total CD3⁺ population (middle panel), IL-22⁺ cells of total SCART2⁺γδ⁺ population (lower panel). *n* = 4 to 18 mice, analyzed in 3 independent experiments. All statistical analysis were performed using student's *t*-test, *p* < 0.05 is significant.

Since CD4⁺Foxp3⁺ Treg cells, SCART2⁺γδ T17-cells as well as RORγt⁺ ILCs have been shown to regulate intestinal homeostasis, we analyzed the population of these immune cells using FACS analysis. CD103⁺CD11b⁻ DCs are known to express high levels of the vitamin A metabolizing enzyme ALDH1A2 which converts vitamin A to retinoic acid [301]. Retinoic acid is important in priming CD4⁺Foxp3⁺ T cells [302], hence we would like to investigate if depletion of CD103⁺ DC subsets would result in decreased CD4⁺Foxp3⁺ T-cells in the LP. Our data clearly show there is no significant difference in percentage of CD4⁺Foxp3⁺ T-cells in both Clec9A⁻ and Clec4a4-DTR mice (figure 3.18A), indicating CD4⁺Foxp3⁺ Treg cells do not have a role in mediating the two opposing phenotypes observed in Clec9A⁻ and Clec4a4-DTR within this early time-point. CD103⁺ DCs have also been shown to express the pro-inflammatory cytokine IL-23 which is important in mediating IL-22 production by RORγt⁺ ILCs and γδ⁺ T-cells [303]. We speculate, depletion of CD103⁺ DCs would result in impaired IL-22 response by RORγt⁺ ILCs and γδ⁺ T-cells. However, from our data, no difference in population of RORγt^{GFP} ILCs as well as their production of IL-22 were found in Clec4a4⁻ and Clec9A-DTR (figure 3.18B). Similarly in the γδ⁺ T-cell fraction, no difference in terms of γδ⁺ T-cells, SCART2⁺ γδ⁺ T-cells and their production of IL-22 was observed in Clec9A⁻ and Clec4a4-DTR. Taken together, our results suggests LP CD4⁺Foxp3⁺ Treg cells, RORγt⁺ ILCs and γδ⁺ T-cells were not affected at day 4 colitis in the absence of either DC subset.

3.4.2 Ablation of CD103⁺ DC subset in Clec9A- and Clec4a4-DTR mice differentially affects colonic epithelial cell expression of anti-microbial peptides genes.

The colonic epithelium plays a crucial role in maintaining an effective barrier against commensals and dietary antigens. Proper functioning of this barrier is important in ensuring commensals and food antigens do not react with the dense underlying network of immune cells in the lamina propria, leading to uncontrolled inflammation. The contribution of DCs in maintaining this important epithelial barrier has not been demonstrated. Since depletion of CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs did not have any effect on the populations of LP CD4⁺Foxp3⁺ Treg cells, RORγt⁺ ILCs and γδ⁺ T-cells during early inflammation (figure 3.18), we speculate DCs directly interact with the epithelia to contribute to intestinal barrier homeostasis. The absorptive epithelium as well as specialized secretory cells of colonic epithelium express key anti-microbial peptides such as REG3γ, REG3β, S100A8, S100A9 and RELM-β which are important for containment of commensals to the lumen [306-308]. In this section, the expression of these key anti-microbial peptides were analyzed. To achieve this, we isolated total RNA from steady-state as well as day 4 DSS-treated WT, Clec9A- and Clec4a4-DTR distal colonic epithelium. Total RNA was then reverse-transcribed to cDNA and normalized using *hprt* primers.

Interestingly, we observed dramatic up-regulation of *Reg3β*, *Reg3γ*, *S100a8*, *S100a9* as well as *Relm-β* in colonic epithelium of Clec4a4-DTR, but not in WT and Clec9A-DTR (figure 3.19A). These anti-microbial peptides were observed to be up-regulated in WT mice during DSS treatment. However, Clec9A-DTR showed only low or undetectable expression (figure 3.19A). Up-regulation of these anti-microbial peptides were higher in DSS-treated Clec4a4-DTR compared to steady state Clec4a4-DTR. To further validate these observations, we analyzed distal colons for the expression of REG3γ and REG3β using immunofluorescence. Consistent with the semi-quantitative PCR analysis, REG3γ and REG3β were dramatically up-regulated in colonic epithelia of steady-state and DSS-treated Clec4a4-DTR while those of Clec9A-DTR showed lowest levels of expression in comparison to WT during early DSS-induced inflammation (figure 3.19B-C).

Fig. 1 Differential expression of epithelium anti-microbial peptides. (A) Analysis of *Reg3β*, *Reg3γ*, *S100a8*, *S100a9*, *Relm-β*, and *Hprt* expression in colon tissue. (B) Analysis of *Reg3β* expression in colon tissue. (C) Analysis of *Reg3γ* expression in colon tissue. All images were taken from colon tissue sections. Scale bars represent 100 μm.

Reg3γ, *S100a8*, *S100a9* and *Relm-β* genes in IECs isolated from steady-state and day 4 DSS-treated WT, Clec9A- and Clec4a4-DTR distal colons using semi-quantitative RT-PCR. Gene expressions are normalized to *Hprt*. (B) Representative confocal fluorescence images of REG3β expression (green) on distal colonic epithelium of steady state and day 4 DSS-treated WT, Clec9A- and Clec4a4-DTR. (C) Representative confocal fluorescence images of REG3γ expression (green) on distal colonic epithelium of steady state and day 4 DSS-treated WT, Clec9A- and Clec4a4-DTR. Confocal sections were co-stained with nuclear stain DAPI (blue). Images were acquired under same settings. 20X magnification. Scale bars denote 50μm. (Figure A: Contributed by Dr Piotr Tetlak and Dr Loh Shuk Zhen as part of our collaborated effort).

In section 3.3.1, we have demonstrated depletion of CD103⁺CD11b⁻ colonic DCs in Clec9A-DTR exacerbates DSS-induced colitis while depletion of CD103⁺CD11b⁺ colonic DCs in Clec4a4-DTR mice offers resistance to DSS-induced colitis. In this section, we have shown that Clec4a4-DTR mice exhibited dramatic up-regulation of colonic epithelium expression of key anti-microbial peptides which are important in containment of bacterial burden during DSS-induced breach of epithelial integrity, hence protecting mice from colitis. In Clec9A-DTR, expressions of these anti-microbial peptides in colonic epithelia are lower or undetectable in comparison to WT mice during day 4 DSS-induced colitis. This may lead to compromised anti-microbial barrier defense resulting in increased susceptibility to DSS-induced colitis.

3.4.3 Ablation of CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs differentially affects colonic epithelial cell expression of *Il-22ra2*, *Il-18bp* and *Ido-1*.

We have demonstrated in section 3.4.1, IL-22 producing immune cells were not affected in the absence of either DC subsets, during early DSS-induced inflammation. Hence we performed genome-wide microarray analysis on distal colonic IECs isolated from day 4 DSS-treated WT, Clec9A-DTR and Clec4a4-DTR, to study the consequences of depleting either DC subset on IEC genes regulating epithelial barrier functions. Interestingly, our gene-array analysis returned gene hits that were of significant value to our study. In particular, the tryptophan metabolizing enzyme Indoleamine 2, 3-dioxygenase (*Ido1*) and IL-18 binding protein (*Il-18bp*) were highly upregulated in WT mice in response to DSS treatment, but were dramatically down-regulated in Clec9A-DTR. Clec4a4-DTR did not show significant differences in expression of these two genes as compared to WT, however gene expression of the secreted form of IL-22 binding protein (*Il-22ra2*) but not its membrane-bound receptor (*Il-22ra1*), was drastically downregulated in IECs of these mice.

We next validated the gene expression of *Il-22ra1*, *Il-22ra2*, *Il-18bp* and *Ido1* in day 4 DSS-treated colonic epithelium using semi-quantitative PCR (Figure 3.20). IL-22BP is a decoy protein that has been demonstrated to neutralize the protective effects of IL-22 [310-311], leading to compromised epithelial barrier defense. IL-18BP, another decoy protein, has been shown to bind active IL-18 and neutralizing its physiological functions [312]. Blocking the activity of IL-18 in the gut has been shown to protect mice from intestinal inflammation [313-314]. IDO-1, a rate limiting enzyme of L-tryptophan conversion to kynurenine, has been shown to have immune-modulatory effects on the inflammatory state of infectious diseases [315]. We speculate DCs modulate the expression of these inflammatory mediators on colonic epithelial cells to control inflammation.

Membrane-bound IL-22bp receptor (*Il-22ra1*) expression in colonic epithelium was found to be equally expressed in steady-state as well as in day 4 DSS-treated WT, Clec9A- and Clec4a4-DTR. However, the expression of secreted IL-22bp (*Il-22ra2*) was only expressed in the epitheliums of steady state and DSS-treated WT and Clec9A-DTR. In contrast, Clec4a4-DTR epitheliums from both steady state and day 4 DSS treatment, showed complete down-regulation of *Il-22ra2* expression (figure 3.20).

Il-18bp expression was found to be up-regulated in DSS-treated WT and Clec4a4-DTR, suggesting the gut immune-system attempt to counteract the damaging pro-inflammatory effects of IL-18. However, consistent with our observations, colitis-susceptible day 4 DSS-treated Clec9A-DTR failed to up-regulate robust levels of *Il-18bp* expression as seen in WT and Clec4a4-DTR (figure 3.20). Lastly, almost undetectable levels of *Ido1* expression can be observed in steady state WT, Clec9A- and Clec4a4-DTR. DSS treatment for 4 days resulted in dramatic up-regulation of *Ido1* expression in only WT and Clec4a4-DTR colonic epithelium. Again, consistent with our observations, colonic epithelium of Clec9A-DTR failed to up-regulate *Ido1* expression (figure 3.20), suggesting an impairment of immunoregulatory mechanism in the absence of CD103⁺CD11b⁻ DCs.

Figure 3.20. CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DC subsets differentially regulate expression of immune mediators in colonic epithelium. RT-PCR semi-quantitative analysis of *Il-22ra1*, *Il-22ra2*, *Il-18bp* and *Ido1* gene expression from pooled IECs of steady-state and day 4 DSS-treated WT, Clec9A- and Clec4a4-DTR mice. PCR results are representative of 3 independent IEC isolations. (Contributed by Dr Piotr Tetlak and Dr Loh Shuk Zhen as part of our collaborated effort).

Our data clearly showed the up-regulation of the two anti-inflammatory mediators *Il-18bp* and *Ido1*, are important in suppressing excessive colonic inflammation. Failure to up-regulate these mediators led to an increased susceptibility to colitis as observed in Clec9A-DTR. Since CD103⁺ DCs were also known to express IDO1 [316], we ask

the question if the epithelium itself is a major source of IDO1. To investigate this, cDNA from sorted DC subsets used previously for the micro-array analysis in section 3.1.2, were analyzed alongside cDNA of WT steady-state and day 4 DSS-treated colonic epithelial cells, for *Ido1* gene expression. Quantitation of *Ido1* transcript was done using qPCR, normalized to gene-expression of β -actin. We observed *Ido1* to be expressed by colonic CD11c^{hi}MHCII⁺ CD103⁺CD11b⁻ (subset 1), CD103⁺CD11b⁺ (subset 2) and CD103⁻CD11b⁺ (subset 3) myeloid cells. CD103⁺CD11b⁻ subset showed the highest expression (figure 3.21A). No significant changes in *Ido1* gene expression were observed in the three CD11c^{hi}MHCII⁺ myeloid cell subsets sorted from steady-state and day 4 DSS-treated colons. Consistent with our semi-quantitative PCR, *Ido1* gene-expression is nearly undetectable in steady-state colonic epithelial cells. However, gene-expression of *Ido1* is dramatically up-regulated ~450 folds in colonic epithelium, after day 4 DSS treatment (figure 3.21A). The numerical excess of colonic epithelium as well as its high gene expression of *Ido1*, points IECs as the major source of this anti-inflammatory mediator.

We next study the gene-expression of *Ido1* and *Il-18bp* over a six day period of DSS treatment. To achieve this, WT mice were fed with 1% DSS and subsequently colonic IECs were harvested from day 0 to 6. To study the time-point gene expression of *Ido1* and *Il-18bp*, RNA was isolated from isolated IECs and analysed by semi-quantitative PCR. Gene expressions were normalized to *Hprt*. *Ido1* gene-expression showed an increasing trend over the entire 6 day 1% DSS treatment. *Ido1* gene-expression can be detected as early as 24h DSS treatment and steeply increase at day 3 to 4. Levels of *Ido1* transcripts gradually increased from day 4 to 6 (figure 3.21B). Similarly, *Il-18bp* gene-expression showed an increasing trend over the entire 6 day time-points, with transcripts peaking at day 4 DSS treatment (figure 3.21B). This shows the levels of the two *Ido1* and *Il-18bp* transcripts increase gradually as colonic inflammation intensifies. Since Clec9A-DTR failed to up-regulate the expression of these 2 anti-inflammatory mediators during early DSS-induced colitis, this may explain the susceptibility of colitis observed in this DTR mouse strain.

Figure 3.21. Colonic epithelial cells are the major producers of *Ido1* during inflammation. (A) *Ido1* gene-expression of sorted CD103⁺CD11b⁻ (DC 1), CD103⁺CD11b⁺ (DC 2), CD103⁻CD11b⁺ (DC 3) subsets and isolated colonic IECs (EC) in steady-state and day 4 DSS treated colons (EC, black bar) $n=3 \pm$ SEM. (B) Time-course of *Ido1* and *Il-18bp* expression over 6 day 1% DSS treatment. Colonic IECs were isolated from 3 distal colons harvested daily for 6 days and analyzed for *Ido1* and *Il-18bp* gene-expression. (Figure 3.21A and B was kindly provided by Dr Piotr Tetlak as part of our collaborated efforts).

Since Clec4a4-DTR also depletes CD11c^{int}MHCII⁺CX3CR1^{hi} LP macrophages (Figure 3.8), we rule out the possibility of this non-specific depletion causing the dramatic up-regulation of anti-microbial peptides (figure 3.19) and down-regulation of *Il-22bp* (figure 3.20). To achieve this, CD169-DTR used previously to deplete CD11c^{int}MHCII⁺CX3CR1^{hi} LP macrophages (Figure 3.9) were included as a control in our gene-expression analysis of anti-microbial peptides and immune mediators. WT and CD169-DTR were subjected to a similar 2% DSS regiment as Clec4a4-DTR, for 4 days and DT injections were given at days -1, 0 and 3. Distal colonic IECs were isolated at day 4, and analyzed for expression the genes as mentioned. Our results clearly show that depletion of CD11c^{int}MHCII⁺CX3CR1^{hi} LP macrophages in CD169-DTR did not result in dramatic up-regulation of anti-microbial peptide genes *Reg3 β* , *Reg3 γ* , *S100a8*, *S100a9* and *Relm- β* , and down-regulation of *Il-22bp* as observed in Clec4a4-DTR (figure 3.22). In addition, CD169-DTR developed exacerbated colitis unlike observed in Clec4a4-DTR (figure 3.14). This suggests the perturbation of gene expressions analysed above is solely contributed by loss of CD103⁺CD11b⁺ DC subset, and exacerbation of colitis in CD169-DTR is mediated by an entirely different mechanism.

	Steady-state		Day 4 DSS	
	WT	CD169-DTR	WT	CD169-DTR
1				
<i>Reg3β</i>				

Figure 3.22. Depletion of CD11c^{int}MHCII⁺CX3CR1^{hi} LP macrophages did not result in differential gene-expression of anti-microbial peptides and immune mediators as observed in Clec4a4-DTR. Semi-quantitative PCR analysis of gene expression for anti-microbial peptides (*Reg3β*, *Reg3γ*, *S100a8*, *S100a9* and *Relm-β*) and immune mediators (*Ido1*, *Il-18bp*, *Il-22ra1* and *Il-22ra2*) from distal colonic IECs isolated from pooled steady-state and day 4 DSS treated WT and CD169-DTR.

3.4.4 Clec9A⁺ DCs support LP and IEL CD8⁺ T-cell production of IFN- γ resulting in up-regulation of *Ido1* and *Il-18bp* in IECs during early DSS-induced colitis.

It was reported IDO1 and IL-18BP expression are induced by IFN- γ [317-318], and the depletion of CD103⁺CD11b⁻ DCs in Clec9A-DTR leads to dramatic down-regulation of both mRNA transcripts in the epithelium during early DSS-induced colitis (figure 3.20). We investigate if CD103⁺CD11b⁻ DCs play a role in controlling colonic expression *Ido1* and *Il-18bp* via an IFN- γ dependent mechanism. To achieve this, we first analyzed if colonic IECs are responsive to IFN- γ . Using semi-quantitative PCR, we tested a colonic epithelial cell-line (CMT-93) and isolated colonic IECs, for the expression of interferon- gamma receptor *Ifngr*. We also stimulated CMT-93 colonic cell line with exogenous IFN- γ to observe for up-regulation of *Ido1* and *Il-18bp*. Here we report the positive expression of interferon- γ receptor gene (*Ifngr*) on colonic IECs as well as in CMT-93 cell-line (figure 3.23A). Stimulation of CMT-93 cells with exogenous IFN- γ , resulted in dramatic up-regulation of *Il-18bp* and *Ido1* after 24h, indicating colonic IECs are responsive to IFN- γ (figure 3.23B).

Figure 3.23. Colonic IECs are responsive to IFN- γ and up-regulates *Il-18bp* and *Ido1* upon exposure. Semi-quantitative PCR analysis of (A) Freshly isolated distal colonic IECs and CMT-93 colonic epithelial cell line analyzed for interferon- γ receptor *Ifngr* gene-expression (B) CMT-93 cells stimulated with exogenous IFN- γ (100 U/ml) *in vitro* for 24h resulting in up-regulation of *Il-18bp* and *Ido1*. All gene expressions were normalized to *Hprt*. (Figures solely contributed by Dr Piotr Tetlak).

We next identify the cellular sources of IFN- γ . Since tissue-resident CD8⁺ T-cells have been shown to mediate their protective functions through secretion of IFN- γ in

response to local physical and microbial insults [319-321], we next investigated if IFN- γ production by tissue-resident LP and IEC CD8⁺ T-cells are affected during depletion of CD103⁺CD11b⁻ DCs. Using percoll-gradient, we isolated colonic LP and IEC CD45⁺ leukocytes, from day four DSS-treated WT and Clec9A-DTR mice. Clec9A-DTR mice were i.p injected with 20ng/g bwt DT given at days -1, 0 and 3. We then stimulated cell isolates in PMA/ionomycin for 1h following Brefeldin A treatment for another 2h. Stimulated cells were then stained for CD45, CD4, CD8, $\gamma\delta$ and intracellular IFN- γ and analyzed by FACS.

In our Clec9A-DTR mice, it is clear that LP CD4⁺ and CD8⁺ T cells exhibited significantly lower IFN- γ response in the absence of CD103⁺CD11b⁻ DCs ($p < 0.05$, $p < 0.001$ respectively) (figure 3.24A). A similar trend was also observed in IEL tissue-resident TCR $\gamma\delta$ ⁻CD8⁺ T-cells ($p < 0.001$). However, we do not see any significant changes in terms of IFN- γ production in other IEL T-cell subsets (figure 3.24B). The impairment in production of IFN- γ by these T-cell subsets could explain the lack of IFN- γ -dependent *Ido1* and *Il-18bp* expression in Clec9A-DTR colonic epithelium.

Since IFN- γ production by CD8⁺ T cells and other lymphocytes is dependent on IL-12 and IL-15 [322-324], and depletion of CD103⁺CD11b⁻ DCs resulted in significant impairment of IFN- γ production by CD8⁺ T cells, we speculate CD103⁺CD11b⁻ DCs to be the source of these cytokines. We next analyzed gene-expression of *Il-12p35*, *Il-15* and *Il-23p19* using qPCR on FACS-sorted colonic myeloid cell subsets (CD103⁺CD11b⁻, CD103⁺CD11b⁺ and CD103⁻CD11b⁺). As expected *Il-12p35* and *Il-15* were observed to be highly expressed by only CD103⁺CD11b⁻ DC subset while *Il-23p19* is highly expressed by CD103⁺CD11b⁺ DCs (figure 3.25). Taken together, we conclude CD103⁺CD11b⁻ DCs are crucial in inducing IFN- γ response in CD8⁺ IEC and LP resident T-cells during DSS-induced damage, leading to up-regulation of *Ido1* and *Il-18bp* in colonic epithelium. The up-regulation of *Ido1* and *Il-18bp* in colonic epithelium is an important mechanism in controlling colonic inflammation.

Figure 3.24. Depletion of CD103⁺CD11b⁻ DCs resulted in impairment of IFN- γ response in IEC and LP resident T-cells. (A) Colonic LP leukocytes from day 4 DSS-treated WT and Clec9A-DTR were analyzed for CD4⁺ and CD8⁺ T-cell production of IFN- γ . Clec9A-DTR showed impairment in both CD4⁺ and CD8⁺ T-cell IFN- γ production (B) Colonic IEL from day 4 DSS-treated WT and Clec9A-DTR were analyzed for TCR $\gamma\delta$ ⁺ and CD8⁺ T-cell production of IFN- γ . Clec9A-DTR showed impairment of CD8⁺ T-cell IFN- γ response but no significant changes were observed in other T-cell populations. Leukocytes were stimulated with PMA/Ionomycin and Brefeldine A for a total of 3 hours. ($n = 5$ per group $\pm$ SEM, representative of 2 independent experiments). Statistical analysis were performed using student's t -test. (Significance: * $p < 0.05$, *** $p < 0.001$).

Figure 3.25. Analysis of cytokine gene-expression of sorted colonic $CD11c^{hi}MHCII^+$ myeloid cell subsets. qPCR analysis of sorted colonic myeloid cell subsets; $CD103^+CD11b^-$ DCs (black bars), $CD103^+CD11b^+$ DCs (shaded bars) and $CD103^-CD11b^+$ macrophages (white bars). $CD103^+CD11b^-$ DCs were the highest in expression of *Il-12* (*Il-12p35*) and *Il-15*, while $CD103^+CD11b^+$ DCs were the highest in expression of *Il-23* (*Il-23p19*). Gene-expressions were normalized to B-actin mRNA. (Figures were provided by Dr Piotr Tetlak as part of our collaboration work).

3.4.4.1 Depletion of CD103⁺CD11b⁻ DCs results in down-regulation of IFN- γ -dependent gene-expression in the colonic epithelia during acute DSS-induced inflammation.

Depletion of CD103⁺CD11b⁻ DCs in Clec9A-DTR have been shown to result in impairment of IFN- γ response in colonic epithelium leading to failure to up-regulate IFN- γ -dependent anti-inflammatory mediators such as *Ido1* and *Il-18bp* (figure 3.20). This finding suggests besides the function of IECs as a physical barrier against commensals, it also responds to signals from dendritic cells to control inflammation. Since IFN- γ is important in mediating resistance against microbial insults [325-326], we next analyzed the expression of other IFN- γ -induced genes that may have roles in protecting the gut and controlling inflammation. To achieve this, total RNA were extracted from steady-state WT, day 4 2% DSS-treated WT and Clec9A-DTR distal colonic epithelium. RNA samples were then processed for micro-array analysis as described in materials and methods.

Remarkably, IECs from 4 day DSS-treated WT mice showed up-regulation of IFN- γ -induced genes such as IFN- γ -induced GTPases (*Gvin1*, *Gbp4*, *Igtp*, *ligp1*), IFN- γ -induced proteins (*Ifit1*, *Ifit2*, *Ifit3*, *Ifit44*), IFN- γ -induced regulatory factors (*Irf1*, *Irf7* and *Irf9*), IFN- γ -induced MHC class II related proteins (*H2-DMb1*, *H2-Ab1*, *H2-Aa*, *H2-Eb1*, *CD74*) as well as our previously identified anti-inflammatory proteins (*Ido-1* and *Il-18bp*) (figure 3.26). IFN- γ is thought to mediate its protective functions through inducing over 200 genes [327], of which mostly of unknown functions. One of these genes include *Igtp*, which has been shown to mediate resistance against *Toxoplasma gondii* infection [328]. The up-regulation of MHCII peptides on epithelium has been shown to protect mice from colitis [329].

However, in Clec9A-DTR, all the above mentioned IFN- γ -induced genes were not up-regulated as observed in WT mice during DSS treatment (figure 3.26). This highlights the important role of CD103⁺CD11b⁻ DCs in controlling IFN- γ -dependent epithelial barrier defense function and inflammation.

Figure 3.26. Depletion of CD103⁺CD11b⁺ DCs in Clec9A-DTR resulted in impaired up-regulation of IFN- γ -induced genes in colonic epithelium.Heat map of selected IFN- γ -induced genes expressed by colonic IECs isolated from steady-state WT, day 4 DSS-treated WT and Clec9A-DTR mice ($n=3$). (Data was generated in collaboration with Dr Loh Shuk Zhen).

3.4.5. Depletion of CD103⁺CD11b⁻ DCs results in profound reduction of Mucin-2 production in affected colons.

Mucins are heavily glycosylated proteins that form a gel coat on epithelial barrier surfaces. On the luminal surfaces of intestines, this mucus coating prevents commensals from being in contact with the epithelium and the underlying immune cells, hence preventing unnecessary immune responses from developing. Another function of this mucus gel is the ability to retain anti-microbial peptides secreted by IECs and Paneth cells, important for keeping the inner mucus layer sterile [330-331]. Mucin-2 (MUC2) is the most abundant of all mucins in gut [332] and mice deficient of MUC2 expression has been shown to develop spontaneous colitis [333]. To understand the mechanism of Clec9A-DTR increased susceptibility to DSS-induced colitis, we next analyzed if depletion of CD103⁺CD11b⁻ DCs affects this protective mucous layer. To achieve this, histological colon sections from day 4 DSS-treated WT, Clec4a4-DTR, Clec9A-DTR, were analyzed for total polysaccharides and mucins using periodic-acid Schiff (PAS) staining. Day 4 DSS-treatment time-point was chosen where no obvious epithelial damage can be observed. PAS staining procedure was performed as directed by manufacturer's manual. In WT sections, PAS staining clearly revealed distribution of total mucins which are localized to epithelial surfaces, colonic crypts and goblet cells. Interestingly, depletion of CD103⁺CD11b⁻ DCs in Clec9A-DTR but not CD103⁺CD11b⁺ DCs in Clec4a4-DTR resulted in reduction of total mucins in colons. This reduction is apparent within the medial and distal parts of colons (figure 3.27A).

We next analyzed if MUC2, is affected as a consequence of depleting CD103⁺CD11b⁻ DCs. Cryosections were obtained from day 4 DSS-treated WT and Clec9A-DTR distal colons. Following acetone-fixation, sections were then immunostained with rabbit monoclonal antibodies for MUC2. Sections were then labelled with anti-rabbit FITC-conjugated secondary antibodies and analyzed by confocal microscopy. Images were acquired at similar settings. Consistent with the observations in PAS staining, distal colon sections from Clec9A-DTR exhibited lower abundance of MUC2 (figure 3.27B, upper panel). MUC2 was highly expressed in goblet cells of DSS-treated WT distal colons while in Clec9A-DTR, MUC2 expression is highly reduced, marked by diminishing MUC2⁺ fluorescence intensities (figure 3.27B, lower panel).

Figure 3.27. Depletion of CD103⁺CD11b⁺ DCs resulted in reduction of secreted mucins. Representative images of (A) PAS staining done on day 4 DSS-treated WT and Clec9A-DTR colon sections. Polysaccharides and mucins were stained red. Colon sections were counterstained green. Reduction in total polysaccharides and mucins can be observed in medial and distal sections of day 4 DSS-treated Clec9A-DTR colons ($n=2$). (B) Confocal images of day 4 DSS-treated WT and Clec9A-DTR colon sections immunostained with anti-MUC2 mAB (green) and counterstained with DAPI (blue). Expression of MUC2 by goblets cells lining the epithelium (upper-panel, 20x magnification). Close-up view of MUC2 production by goblet cells (lower panel, 60x magnification). All scale bars, 50 μ m.

Next, we analyzed the distribution and expression of another goblet cell-derived product, trefoil peptide 3 (TFF3), which is known to be crucial for maintaining structural integrity of the mucus layer [334-335]. Cryosections were obtained from swiss-rolled colons of day 4 DSS-treated WT and Clec9A-DTR. Acetone-fixed sections were then immunostained with rabbit anti-TFF3 monoclonal antibodies and secondary-labelled with FITC-conjugated anti-rabbit monoclonal antibodies. Immunostained sections were then analyzed for TFF3 expression using fluorescence microscopy. No differences in terms of distribution or expression of TFF3 were observed in DSS-treated Clec9A-DTR when compared to DSS-treated WT (figure 3.28). Since TFF3 expression has been established to be directly regulated by TLR2 receptors expressed on IECs, which recognizes bacterial peptidoglycans [336], depletion of CD103⁺CD11b⁻ DCs may have no effect on TFF3 expression.

Figure 3.28. Expression of TFF3 by goblet cells is not affected by depletion of CD103⁺CD11b⁻ DCs. Representative images of fluorescence staining for TFF3 (green) of proximal, medial and distal colonic sections from day 4 DSS-treated WT and Clec9A-DTR. TFF3 is expressed by goblet cells lining the apical epithelium and crypts. (*n*=2). Magnification 20X. Scale bar, 50µm.

3.4.6 Exacerbation of DSS-induced colitis severity in Clec9A-DTR can be reverted through induction of IFN- γ -dependent response in colonic epithelia.

We have demonstrated in section 3.4.3, that depletion of colonic CD103⁺CD11b⁺ DCs in Clec9A-DTR resulted in impairment of IFN- γ production by CD8⁺ T-cells residing in LP and IEC, leading to poor induction of IFN- γ -inducible genes such as *Ido1* and *Il-18bp*. IDO1 and IL-18BP have been shown to have anti-inflammatory properties [313-315]. Hence we ask the question if the susceptibility of DSS-induced colitis observed in Clec9A-DTR is mediated by the lack of *Ido1* and *Il-18bp* expression in colonic IECs. To investigate this question, WT and Clec9A-DTR were subjected to a 2% DSS regiment as illustrated in figure 3.11A.

Figure 3.29. Treatment of Clec9A-DTR with ISS-ODN limits the severity of DSS-induced colitis. WT and Clec9A-DTR were given 2% DSS in drinking water for 7 days and fresh drinking water for subsequent 7 days. Both groups were injected i.p 10ug ISS-ODN at day 0 and 4.(A) Body-weight loss monitoring of ISS-ODN-treated WT and Clec9A-DTR during DSS treatment (n=8 per group from 2 independent experiments). (B) Day 2 serum IFN- γ levels from ISS-ODN-treated WT and Clec9A-DTR quantitated by ELISA (n=8 per group from 2 independent experiments). (C) *Ido1* gene-expression in day 4 DSS-treated WT and Clec9A-DTR, with and without ISS-ODN treatment, quantitated using semi-quantitative PCR. Gene expressions of *Ido1* normalized to *hprt*. Results representative of 3 independent experiments. (Figures A and B were kindly provided by Yolanda Setiagani and Dr Piotr Tetlak as part of our collaborated efforts).

In addition, a separate group of WT and Clec9A-DTR were given i.p administration of 10ug immunostimulatory oligonucleotides (ISS-ODN: 5'-TGACTGTGAACG TTCGAGATGA-3') at day 0 and 4, which have been shown to trigger IFN- γ -response and to limit disease severity in experimental colitis [339-340]. Bacterial DNA and its synthetically derived ISS-ODNs stimulate macrophages, monocytes, dendritic cells, and natural killer cells to secrete cytokines such as IL-6, IL-12, and IFNs [394]. We report the treatment of both mouse groups with ISS-ODN was efficient in up-regulating IFN- γ as observed in serum at day 2 (figure 3.29B) and its gene-expression in IECs of Clec9A-DTR at day 4 DSS treatment (figure 3.29C). Most importantly, it is clear that treatment with ISS-ODN reverted the colitis-prone phenotype observed in Clec9A-DTR since they behaved similarly to WT in response to DSS treatment (figure 3.29A). Taken together, our data strongly suggests the importance of CD103⁺CD11b⁻ DCs in controlling colonic inflammation through regulation of IFN- γ response in IECs hence limiting severity of inflammation.

3.4.7 Conclusion

To begin unraveling the mechanism of how colonic CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DC subsets control colonic inflammation, we first analyzed populations of LP immune cells such as CD4⁺Foxp3⁺ Tregs cells, SCART-2⁺ $\gamma\delta$ T17-cells as well as ROR γ t⁺ ILCs for the production of IL-22 cytokine, in the absence of either DC subset. Surprisingly, depletion of either DC subset during early inflammation did not affect the numbers of CD4⁺Foxp3⁺ Tregs cells or the ability to produce IL-22 by SCART-2⁺ $\gamma\delta$ T17-cells and ROR γ t⁺ ILCs. This is probably due to the early time point of analysis (day 4 DSS). We next studied the expression of anti-microbial peptides and inflammatory mediators in IECs of Clec9A- and Clec4a4-DTR during early inflammation. Remarkably, depletion of CD103⁺CD11b⁻ DCs in Clec9A-DTR resulted in lower gene-expression of anti-microbial peptide genes *Reg3 γ* , *Reg3 β* , *S100a8* and *S100a9* as well as an array of IFN- γ -induced genes including anti-inflammatory *Ido1* and *Il-18bp*. Importantly, restoration of *Ido1* expression using ISS-ODN, reverted the colitis-prone phenotype of Clec9A-DTR. FACS analysis revealed CD8⁺ T-cells from LP and IECs were impaired in production of IFN- γ . PAS-stained histological sections of Clec9A-DTR medial and distal colon section revealed lower abundance of total polysaccharides and mucins. Notably fluorescence microscopy

showed significant decrease in MUC2 expression by goblet cells. Taken together, our data highlights the important role of CD103⁺CD11b⁻ DCs in maintaining functional epithelial barrier defense through regulating IEC secretion of anti-microbial peptides and mucins. In addition CD103⁺CD11b⁻ DCs regulate production of IFN- γ by CD8⁺ T cell in IECs and LP, which are important in inducing IEC expression of anti-inflammatory mediators such as *Ido1* and *Il-18bp* to control inflammation. On the other hand, depletion of CD103⁺CD11b⁺ DCs in Clec4a4-DTR, leads to dramatic increase of anti-microbial peptide genes *Relm- β* , *Reg3 γ* , *Reg3 β* , *S100a8* and *S100a9* in IECs and down-regulation of IL-22 neutralizing *Il-22bp*, resulting in resistance to DSS-induced colitis. The up-regulation of REG3 γ and REG3 β in IECs of Clec4a4-DTR have been confirmed by confocal immunofluorescence analysis. Taken together, we conclude CD103⁺CD11b⁺ DCs play a pro-inflammatory function during pathogenesis of colitis.

4. Discussion

DCs have been implicated to play an important role in orchestrating appropriate host responses towards harmless antigens, commensals and pathogens hence contributing to maintenance of intestinal immune homeostasis [341]. In the context of DSS-induced inflammation, it has been demonstrated using CD11c-DTR mice, depletion of CD11c⁺ DCs prior to DSS exposure, attenuated DSS-induced colitis [295]. This result shows DCs play a role in development of colitis. However, the use of CD11c-DTR which ablates total colonic DCs proved to be a limitation in dissecting the role of different DC subsets in regulating colonic inflammation. FACS analysis of colonic CD11c^{hi}MHCII⁺ myeloid cells revealed 3 subsets consisting of CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs and a third macrophage CD103⁻CD11b⁺ subset. In addition, we also highlighted the CD11c^{int}MHCII⁺ myeloid cells consists of a heterogeneous population of CX3CR1⁻CD64⁻ cells, CX3CR1^{int}CD64^{int} monocytes and CX3CR1^{hi}CD64^{hi} macrophages (Figure 3.1). Our transcription profile analysis of sorted colonic CD11c^{hi}MHCII⁺ myeloid cells subsets confirmed colonic LP bona fide DCs consists of 2 subsets namely; CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs. CD103⁺CD11b⁻ DCs in particular express the transcription factors *Batf3*, *Irf8* and *Id2* while CD103⁺CD11b⁺ DCs express markers similar to CD103⁺CD11b⁻ DCs, albeit at lower levels such as *Flt3*, *Irf5* and *Id2*. Moreover, CD103⁺CD11b⁺ DCs exclusively express the transcription factor *Irf4* (Figure 3.2D). Similar to CD8⁺ lymph node resident DCs, CD103⁺CD11b⁻ colonic DCs are dependent on *Batf3*, *Irf8* and *Id2* for their development [281, 342, 368-371]. In contrast, CD103⁺CD11b⁺ DCs which form the minor DC subset in the colon, develop independently of *Batf3*, *Irf8* and *Id2* [342, 372] but requires *Irf4* for development [373]. We have shown here that these two distinct DC subsets differ not only in their developmental pathways but may also have their own specialized physiological functions in regulating colonic inflammation.

Our transcription profile analysis of sorted colonic DC subsets have identified 2 C-type lectins; Clec9A (DNDR1) and Clec4a4 (DCIR2) which are respectively expressed on CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs (Figure 3.2D). To begin unraveling the role of these two distinct DC subsets in regulating colonic

inflammation, Clec9A-DTR and Clec4a4-DTR mice capable of depleting CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DC subsets respectively were generated by our lab. Efficient depletion of target colonic DC subsets in both DTR mice was observed. To study the roles of CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs in regulating colonic inflammation, Clec9A- and Clec4a4-DTR were subjected to DSS-induced model of colitis. WT mice as a comparison, during the 2% DSS regiment developed mild clinical signs of colitis which includes body weight loss, presence of fecal occult blood, colon shrinkage, increased intestinal permeability and mucosal erosion (Figure 3.11). Remarkably, Clec9A-DTR suffered dramatic exacerbation of colitis marked by severe body weight loss, increased in fecal occult blood scores, colon shrinkage, intestinal permeability and mucosal erosion as compared to WT. Surprisingly, Clec4a4-DTR when subjected to the same (2%) DSS regiment, were found to be resistant to colitis (Figure 3.11). In fact, Clec4a4-DTR mice developed only mild colitis even when given a higher DSS concentration of 5% (Figure 3.11a). Taken together, these results highlight for the first time, that CD103⁺CD11b⁻ DCs protects mice from exacerbated colitis while CD103⁺CD11b⁺ DCs have a more pro-inflammatory role during colonic inflammation. The exacerbated colitis exhibited by Clec9A-DTR as a consequence of depleting CD103⁺CD11b⁻ DCs, contradicts with the phenotype observed in *Batf3*^{-/-} mice [342] since these mice which lack CD103⁺CD11b⁻ DCs did not exhibit symptoms of exacerbated colitis as observed in Clec9A-DTR. Unlike our Clec9A-DTR which conditionally ablates CD103⁺CD11b⁻ DCs, *Batf3*^{-/-} mice were absent in CD103⁺CD11b⁻ DCs since birth. Since the gut microenvironment plays an essential role in shaping DC functions [343], this may have conditioned other CD11c⁺MHCII⁺ myeloid cell subsets to functionally compensate for the loss of CD103⁺CD11b⁻ DCs, hence a possible reason why *Batf3*^{-/-} mice were not affected in terms of susceptibility to DSS-induced colitis. In addition, *Batf3*^{-/-} mice were subjected to a high dose of 5% DSS which induces severe colitis in WT mice hence may overwhelm the protective properties of CD103⁺CD11b⁻ DCs. Other possible reasons include differences in mouse genetic background (129SvEv vs BALB/c) and differences in gut microbiota.

The anatomical locations of the two DC subsets may also account for their differences in physiological functions (Figure 3.10). Clec9A⁺CD103⁺CD11b⁻ DCs residing in the colonic LP in close proximity to the crypt and absorptive epithelium may be

conditioned by epithelium-derived factors such as TSLP, TGF- β and dietary RA to induce tolerogenic DCs [343,344]. Clec4a4⁺CD103⁺CD11b⁺ DCs which were absent in colonic lamina propria but were found localized in innate lymphoid follicles and other colonic lymphoid tissues, were not directly exposed to these factors hence may escape conditioning resulting in acquisition of pro-inflammatory functions.

The clear opposing phenotypes observed as a consequence of depleting CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DC subsets in our DSS-induced model of colitis prompted us to study the mechanistic details of how these two DC subsets mediate their functions in controlling gut inflammation and homeostasis. The ability of colonic CD11c⁺MHCII⁺ myeloid cells in maintaining gut immune homeostasis is not an entirely new concept. Others have demonstrated the IL-23 secreting CD103⁺CD11b⁺ DCs as well as the IL-10 conditioned CX3CR1⁺ gut macrophages do have important roles in maintenance of gut defense and homeostasis [303, 345-347]. IL-23 secretion by CD103⁺CD11b⁺ DCs stimulates production of gut protective IL-22 by a variety of immune cells such as ROR γ t⁺ ILCs, LT α i cells, NK cells, T-cells subsets such as Th17 and $\gamma\delta$ T-cells [300]. IL-22 secretion by these cellular sources stimulate epithelial production of anti-microbial peptides Reg3 γ , Reg3 β , S100A8, S100A9 and β -defensins, resulting in containment of Gram (+) and Gram (-) gut commensals [300, 348-349]. On the other hand, IL-10 conditioned CX3CR1⁺ gut macrophages were known to maintain intestinal integrity since a reduction of these macrophages in CX3CR1^{-/-} mice resulted in increased epithelial translocation of luminal commensals to the mesenteric lymph nodes leading to priming of inflammatory T_H17 cells [277]. In this study, we demonstrate the important role of CD103⁺CD11b⁻ and CD103⁺CD11b⁺ DCs, in orchestrating innate gut defense and control of intestinal inflammation.

An intact and functional mucosal barrier composed of a layer of IECs is important in keeping commensals and dietary antigens from reacting with a dense network of underlying immune cells. Failure of maintaining this barrier results in translocation of commensals into the LP leading to IBD. Maintenance of this mucosal barrier involves a complex interplay of both immune cells such as DCs, ILCs, T-cell subsets and non-immune cells such as IECs [350]. IECs express an arsenal of pattern-recognition receptors such as TLRs and NLRs [351-352] which enables the sensing and integration of microbial signals resulting in expression of anti-microbial peptides and

mucins to further reinforce this mucosal barrier [353-357]. Since it has been documented that IECs respond to IL-22 secreted by underlying immune cells such as $\text{ROR}\gamma\text{t}^+$ ILCs, LTi cells, NK cells and T-cells, resulting in up-regulation of anti-microbial peptides, this adds another layer of modulation to maintain a functional epithelial barrier [300]. IL-22, a key cytokine whose expression is dependent on IL-23 secreted by $\text{CD103}^+\text{CD11b}^+$ DCs, has been shown to protect the gut from DSS-induced colitis [358]. These findings highlight the central role of DCs in supporting a functional epithelial barrier important in maintenance of intestinal immune homeostasis.

We next studied the regulation of both colonic LP $\text{CD103}^+\text{CD11b}^-$ and $\text{CD103}^+\text{CD11b}^+$ DCs on the epithelial barrier responses during inflammation using a DSS-induced model of colitis. Since expression of AMPs by IECs has been shown to be an important mechanism to contain the large numbers of commensals to the lumen [307-309], we analyzed gene expressions of different AMPs in our Clec9A-DTR mice at day 4 DSS treatment. Remarkably, we observed a down-regulation of AMP genes such as *Reg3 γ* , *Reg3 β* , *S100a8* and *S100a9* in IECs of day 4 DSS-treated Clec9A-DTR (Figure 3.19A). Analysis of total mucins, in particular MUC2 by PAS staining and fluorescence microscopy, revealed a decrease in abundance of total mucins in the mucosa and MUC2 expression in goblet cells respectively (Figure 3.26). Taken together, the reduction of AMPs and mucin expression by IECs observed in Clec9A-DTR mice could lead to a compromised epithelial barrier, which may lead to translocation of commensals to the lamina propria resulting in exacerbation of colitis (Illustrated in figure 4.1B). This impairment of epithelial barrier could explain the increased formation of tertiary lymphoid tissues observed in Clec9A-DTR (Figure 3.17) which arise as a compensatory mechanism to augment a compromised immunity similarly observed in $\text{ROR}\gamma\text{t}^{-/-}$ mice lacking in ILCs, when subjected to similar DSS-treatment [298].

In contrast to the phenotype observed in our Clec9A-DTR mice, depletion of $\text{CD103}^+\text{CD11b}^+$ DCs in Clec4a4-DTR protects mice from exacerbated colitis, highlighting the pro-inflammatory role of this DC subset during inflammation. Comparison of different cytokines expressed by these two DC subsets revealed $\text{CD103}^+\text{CD103}^+$ DCs as the major producers of IL-23 whereas IL-12 and IL-15 were

expressed at lower levels by this subset (Figure 3.25). IL-23 is an inflammatory cytokine which when excessively upregulated plays a role in aggravating experimental colitis in mice [367]. In a CD40-induced model of colitis, deficiency in IL-12p35 but not IL-23p19 in a *Rag1*^{-/-} background resulted in colitis marked by increased colonic expression of TNF- α , MCP and IL-6 [36]. This result highlights IL-23 other than promoting colitis-inducing T_H1 and T_H17 responses [256], also drives intestinal inflammation independently of T-cells. At the same time, IL-23 secretion by CD103⁺CD11b⁺ DCs stimulates production of gut protective IL-22 which stimulates epithelial production of anti-microbial peptides resulting in containment of Gram (+) and Gram (-) gut pathogens [300, 348-349]. Hence depletion of IL-23-producing CD103⁺CD11b⁺ DCs would result in less epithelial expression of anti-microbial peptides. Paradoxically, IECs isolated from day 4 DSS-treated Clec4a4-DTR mice showed dramatic up-regulation, instead of down-regulation, of anti-microbial peptides *Reg3 γ* , *Reg3 β* , *S100a8* and *S100a9* gene expression (Figure 3.19). Since up-regulation of these anti-microbial peptides is dependent on IL-22, we speculate an increased local production of IL-22 in the mucosa. However, we observed during early inflammation, depletion of CD103⁺CD11b⁺ DCs in Clec4a4-DTR did not affect IL-22 production by ROR γ t⁺ ILCs as well as SCART2⁺ γ δ T17-cells (Figure 3.18), hence the protective effects of IL-22 through induction of AMPs in IECs, may be mediated by a different mechanism. Genome-wide transcription profile analysis comparing day 4 DSS-treated IECs of WT and Clec4a4-DTR revealed a dramatic down-regulation in gene-expression of a key decoy protein IL-22BP (IL-22RA2) in Clec4a4-DTR (Figure 3.20). IL-22BP has been shown to bind IL-22 and neutralize its biological activity [310-311] hence neutralising the protective effects of IL-22 on the epithelium. Since the expression of *Il-22bp* is completely abrogated in IECs of Clec4a4-DTR, the increased colitis resistance observed in Clec4a4-DTR could possibly be due to increased availability of bio-active IL-22 in the mucosa, leading to dramatic up-regulation of AMPs and protection of mice from colitis (Illustrated in figure 4.1C). However, a study by Huber *et al* demonstrated IL-22 mediates protective effects during early inflammation and pathogenic properties during the resolution of DSS-induced colitis [374]. In line with this finding, colonic *Il-22bp* was shown in this study to be down-regulated to the lowest levels during peak of DSS-induced colitis (day 7 to 8) and then gradually return to higher levels during the resolution phase (day 15 to 21). In addition, *Il-22bp*^{-/-} mice when subjected to similar DSS regiment did not

demonstrate differences in disease severity when compared to WT mice. On the other hand, colons of WT mice showed lowest expression of *Il-22bp* at a relatively late time-point (day 7 to 8 DSS regiment) corresponding to peak disease. Hence this explains why *Il-22bp*^{-/-} mice did not exhibit differences in disease severity in comparison to WT during acute DSS-induced damage since the protective effect of *Il-22bp* downregulation occurs at a later time point. However, *Il-22bp*^{-/-} mice exhibited increased epithelial cell proliferation during the resolution phase of colitis, where in a colitis-associated colon cancer model resulted in increased tumor burden. In an inverse manner to *Il-22bp* expression, *Il-22* expression in colons were highest at day 7 and 8 DSS regiment which correlated with peak of disease. In addition, *Il-22*^{-/-} mice exhibited increased susceptibility to DSS-induced damage with a marked increase in IL-6 production. These observations suggest IL-22 plays a protective role during peak DSS-induced damage, but can be detrimental during resolution phase of disease therefore its effects must be controlled by IL-22BP. We observed clear abrogation of *Il-22bp* in steady-state IECs isolated from Clec4a4-DTR mice, where IECs were unperturbed. Similarly, in acute inflammatory conditions, *Il-22bp* expression was clearly abrogated in IECs isolated from our Clec4a4-DTR from day 4 DSS treatment. This clear depletion of *Il-22bp* occurs at a significantly earlier time-point than what was observed in Huber *et al.*, where expression of *Il-22bp* was lowest at day 7 and 8 correlating with peak of disease. Hence we propose the protective effect of IL-22 was triggered at a much earlier time-point, before the perturbation of IECs, through earlier down-regulation of IL-22BP, hence conferring Clec4a4-DTR mice resistant to DSS-induced inflammation.

Il-22bp expression has been demonstrated to be strongly down-regulated by IL-18 whose production is dependent on both NLRP3 and NLRP6 inflammasomes [374]. The cellular sources of IL-18 includes DCs and IECs [374]. Inflammasomes are large cytoplasmic protein complexes which function as sensors to endogeneous or exogeneous stress. They can be triggered by host-derived factors released during tissue damage, such as hyaluronan, ATP, uric acid and also bacterial ligands [377]. Perturbation of colonic epithelial layer during DSS treatment results in penetrance of bacterial products, which may lead to activation of inflammasomes hence downregulation of IL-22BP. This is supported by the observation that antibiotics treatment prior to induction of DSS-induced colitis resulted in impaired

downregulation of *Il-22bp* [374]. However, in our non DSS-treated Clec4a4-DTR, the downregulation of IEC *Il-22bp* expression was already observed even in the absence of DSS-induced epithelial damage and penetrance of bacterial ligands (Figure 3.19). Thus we propose: 1) Since depletion of CD103⁺CD11b⁺ DCs did not result in downregulation of *Il-18bp* in IECs (which could lead to increased IL-18 inhibition on *Il-22bp* gene expression [374]), downregulation of *Il-22bp* in IECs could be mediated by an IL-18-independent mechanism. This can be supported by the observation that although IL-18 strongly downregulates *Il-22bp* expression, *Il-22bp* expression can also be mildly downregulated to almost half in IL-18^{-/-} mice during day 7 DSS treatment corresponding to peak of disease [374]. 2) CD103⁺CD11b⁺ DCs may exert their function on IECs rendering them unresponsive to bacterial ligands hence resulting in lack of inflammasome-dependent upregulation of IL-18 in IECs. Thus depletion of CD103⁺CD11b⁺ DCs resulted in increased inflammasome-dependent response of IECs to bacterial ligands. This leads to increased IL-18 –dependent downregulation of *Il-22bp* in IECs as observed in our non DSS-treated Clec4a4-DTR.

Analysis of cytokine gene expression in colonic CD11c⁺MHCII^{hi} myeloid cell subsets revealed CD103⁺CD11b⁻ DC subset is the major producer of IL-12, which upon depletion in Clec9A-DTR, resulted in decreased local production of IFN- γ by CD4⁺ and CD8⁺ LP T-cells as well as TCR $\gamma\delta$ CD8⁺ intraepithelial T-cells (Figure 3.24). As a result, genome-wide transcription analysis of IECs isolated from DSS-treated Clec9A-DTR revealed a down-regulation of IFN- γ -induced genes (Figure 3.26). These genes include the anti-inflammatory mediators *Ido1* and *Il-18bp*. IDO1, a rate limiting enzyme of L-tryptophan conversion to kynurenine, has been shown to have immune-modulatory effects on the inflammatory state of infectious diseases [315]. Deficiency in IDO1 has been associated with IBDs [364-365]. IDO1 mediates its immunosuppressive functions through decreasing the bioavailability of L-tryptophan for bacterial growth, and increasing its enzyme-catabolized products such as kynurenines which induce cell-growth arrest and T-cell unresponsiveness [359-360]. Consistent with other studies, we demonstrate DCs, in particular the CD103⁺CD11b⁻ subset, is the major source of IDO1 in steady state (Figure 3.21A) which is essential for maintenance of local immunosuppressive FOXP3⁺ Treg cells [361-363]. However during early inflammation, we observed a dramatic ~450x fold increase in relative gene-expression of *Ido1* in IECs of day 4 DSS-treated WT in comparison to steady-

state, while in DCs, relative expression of *Ido1* were maintained to at most ~55x fold(Figure 3.21A). Considering the numerical excess of IECs, and the dramatic up-regulation of *Ido1* in early inflammation, we are confident to point IECs but not DCs, as the major source of IDO1 which may be important to limit exuberant inflammation and microbial growth.

Il-18bp, another candidate IFN- γ -induced gene observed to be down-regulated in colitis-prone Clec9A-DTR, is yet another IEC-expressed gene whereby its expression is controlled by CD103⁺CD11b⁻ DCs. Since blocking the inflammatory activity of IL-18 by IL-18BP has been shown to attenuate intestinal inflammation [313-314], IL-18 is thus an important cytokine required for development of DSS-induced colitis. Moreover, excessive production of IL-18 through hyperactivation of inflammasomes has been shown to enhance severity to DSS-induced colitis [378]. Surprisingly, it has been shown that *Il-18*^{-/-} mice demonstrated exacerbated colitis rather than resistance, and also increased tumor burden in a colitis-associated colon cancer model [374]. This could be explained since IL-18 is required for the maintenance of epithelial barrier integrity [379], complete absence of this multifaceted cytokine in *Il-18*^{-/-} mice results in a dysfunctional barrier leading to exacerbated colitis. Hence IL-18 seems to exert a dual role in controlling intestinal homeostasis and disease. Consistent with the colitis-prone phenotype of Clec9A-DTR mice, where *Il-18bp* expression in IECs is significantly reduced at early day 4 DSS treatment, coupled with the observation that at the same time frame, IL-18 expression is upregulated after DSS-exposure [314, 380], the deleterious effect of IL-18 is in full effect leading to exacerbated colitis. Interestingly, as discussed previously, since increased bio-availability of local IL-18 as a consequence of downregulation of *Il-18bp*, has been shown to downregulate *Il-22bp* expression in IECs, we did not observe downregulation of *Il-22bp* in IECs of our Clec9A-DTR mice. Therefore, this observation supports the findings from another study [374] which demonstrates *Il-22bp* expression can be regulated by alternative mechanisms.

Other IFN- γ -induced genes down-regulated in Clec9A-DTR mice include MHCII related proteins (Figure 3.26) which when up-regulated in IECs have been shown to protect mice from colitis [329]. IECs are uniquely positioned at the interface between underlying intestinal mucosa and the myriad of food antigens and commensals in the

lumen. As such, they are in an ideal position to regulate T-cell responses to mucosal antigens. It has been suggested, MHCII⁺ IECs contribute to differentiation of Treg cells resulting in maintenance of tolerance. In this study, upon exposure to neoantigen, IECs induced the local expansion of antigen-specific immune-modulatory Treg cells in the LP independently of DCs [375]. The requirement of MHCII expression in IECs to maintain tolerance has been analysed by selectively depleting (class II trans-activator) CIITA in IECs. When these mice were subjected to colitis induced by IL-10 blockade, exacerbated colitis was observed as compared to WT mice. Analysis of T-cell population in the inflamed colon revealed increased local TH1 cells and imbalanced effector T-cell/Treg cell ratios favoring that of effector T cells [329]. In addition, it was also observed MHCII⁺ colonic epithelial cells suppressed CD4⁺ T cell responses stimulated by antigen-presenting cells *in vitro* [329, 376]. While the mechanisms underlying the protective effects of MHCII expression in IECs were poorly understood, these studies suggest MHCII expression in IECs protects the gut from colitis. Hence these results are consistent with the colitis-prone phenotype of our Clec9A-DTR where MHCII-related genes are down-regulated in IECs during early DSS-induced inflammation.

Besides IL-12, IFN- γ -production by CD8⁺ memory T cells has been demonstrated to be upregulated by IL-18 [382, 383]. CD8⁺ DCs have been shown to produce IL-18 which is dependent on the inflammasome NLRC4 [381]. Through stimulation of cytosolic NLRC4 by bacterial flagellin, IL-18 expression by CD8⁺ DCs was sufficient to stimulate non-cognate effector functions on IEL CD8⁺ memory T cells through production of IFN- γ . This is supported by their observation that *Il-18*^{-/-} mice but not *Il-12*^{-/-} mice failed to stimulate IFN- γ in CD8⁺ memory T cells [381]. These findings were relevant to our study since CD103⁺CD11b⁻ DCs were the equivalent mucosal counterparts of lymphoid CD8⁺ DCs. Hence we suggest, CD103⁺CD11b⁻ DCs may also produce IL-18 dependently of NLRC4 after exposure to commensal ligands during DSS-induced IEC damage. This in turn stimulates IFN- γ production by IEC and LP CD8⁺ T cells, resulting in upregulation of IFN- γ -induced genes in IECs, critical for mediating intestinal barrier defense. Similar to IL-22 and IL-18 as discussed above, IFN- γ may also exert pleiotropic effects depending on the context of inflammation. It has been shown, IFN- γ is causatively involved in experimental inflammatory disease in mice, since IFN- γ ^{-/-} mice failed to develop DSS-induced

colitis [366]. At the same time, *in vitro* exposure of epithelial monolayers to IFN- γ resulted in increased tight-junction permeability which weakens epithelial defense leading to IBDs [384]. In contrast IFN- γ has been discussed previously to mediate anti-inflammatory functions by upregulating the expression of MHCII on IECs, which supports local Treg cell expansion in LP independently of DCs [375]. In addition, IFN- γ also mediates anti-inflammatory properties through stimulating Paneth cells to produce AMPs [386], and goblets cells to secrete mucins [385]. Most importantly, restoration of serum IFN- γ in Clec9A-DTR through immune-stimulatory DNA reverted the colitis-prone phenotype observed in Clec9A-DTR (Figure 3.29). These findings reinforced the multiple roles of IFN- γ where it can function both as a pro-inflammatory mediator leading to IBDs [366], and also protective functions during early inflammation.

The secretion of mucins by goblet cells has been demonstrated by Songhet *et al* to be dependent on IFN- γ , since a selective knock-out of IFN- γ R on goblet cells but not on hematopoietic cells resulted in failure of goblet cells to secrete MUC2 during infection with *Salmonella Typhimurium*. This effect leads to accumulations of MUC2 in colonic goblet cells of IFN- γ R^{-/-} mice resulting in exacerbation of disease [385]. Furthermore, IFN- γ -induced MUC2 secretion have been shown to dissociate commensals from colonic mucosa, protecting mice from lethal infectious colitis [387]. The retention of MUC2 in goblet cells of IFN- γ R^{-/-} mice during infection by *Salmonella Typhimurium* suggests IFN- γ induces goblet cell secretion of MUC2 but does not affect transcription of this protein. Since IFN- γ signalling mediates the expulsion of MUC2 contents in goblet cells, and Clec9A-DTR mice exhibited reduced IFN- γ production in the colonic epithelium during DSS-induced inflammation, we expected impaired goblet cell MUC2 secretion in Clec9A-DTR mice. This event would lead to an accumulation of MUC2 in goblet cells of Clec9A-DTR mice at day 4 DSS treatment. Paradoxically, our MUC2-immunostaining revealed decreased MUC2 signal intensities in colonic goblet cells of DSS-treated Clec9A-DTR, indicating MUC2 expulsion rather than the expected accumulation has occurred. However, increased expulsion of mucins to the lumen is unlikely since PAS-stained Clec9A-DTR colon revealed severe reduction of total mucins lining the colonic epithelial crypts. This occurrence can be explained since goblet cells in IFN- γ R^{-/-} mice were completely devoid of IFN- γ stimulation, goblet cells in Clec9A-DTR were able to be

stimulated by IFN- γ , albeit at lower levels due to less production of IFN- γ by IEL CD8⁺ T cells (figure 3.22), hence were still able to secrete mucins. Therefore, the reduction of total secreted mucins lining the epithelium, as a consequence of depleting CD103⁺CD11b⁻ DCs, suggests goblet cells are less efficient in producing and secreting MUC2 during DSS-inflammation. This inefficient MUC2 expulsion would result in accumulation of MUC2 in goblet cells. However, goblet cells in Clec9A-DTR showed a depletion in MUC2 contents instead of accumulation (figure 3.27B, right panel). This suggests goblet cells are impaired in the production of this protein which may arise from a defective alternate mechanism of production. To support this, various studies have demonstrated MUC2 expression is regulated by other mechanisms such as retinoic acid [388-390]. Indeed, CD103⁺CD11b⁻ DCs have been reported to express high levels of the retinoic acid-converting enzyme ALDH1A2 [301] hence they contribute to the local retinoic acid production. We suggest the depletion of CD103⁺CD11b⁻DCs may result in declining local levels of retinoic acid in the mucosa which in turn resulted in less production of mucins by goblet cells.

Our findings highlight the deleterious effects of alterations in cytokine expression and regulation of their biological activities in the mucosa, leads to exacerbated colitis. In fact, dysregulated patterns of cytokine expression in immune cells of lamina propria has been described in patients with IBDs since the mid-1980s to early 1990s [391, 392]. We suggest strategies in treatment of IBDs. First, IL-18 has been shown to augment the secretion of other inflammatory cytokines such as TNF- α and IFN- γ by macrophages [313]. Blocking of IL-18, a cytokine upregulated in APCs and IECs of patients with Crohn's disease, has been shown to be beneficial in many mouse models of acute and chronic colitis [393]. Hence IL-18 can be targeted by therapeutic interventions to block its deleterious effects during treatment of IBDs. Second, IFN- γ has been shown to control goblet cell secretion of MUC2 [385], while retinoic acid induces its transcription and expression [388-389]. Together, exogenous stimulation of IFN- γ production by administration of TLR9 ligands such as CpG oligodeoxynucleotides, and retinoic acid may be used in treatment of IBDs caused by deficiency of mucin production in mucosa. Finally, our results have shown, increased production of AMPs by IECs mediated through down-regulation of IL-22BP, protects mice from colitis. Hence drugs blocking IL-22BP activity could be designed as a strategy to treatment of IBDs. However, careful assessment of the underlying cause of

IBD has to be performed before deciding the type of IL-22-based treatments since this cytokine mediates increased tumor burden in colitis-associated colon cancer mouse model of colitis, but exhibits protective anti-colitic effects in mouse model of acute *Citrobacter rodentium* infections [349].

Our study focusing on epithelial expression of IL-18BP and IL-22BP relied heavily on quantitative mRNA expression presented some drawbacks. This extends to the quantitation of *IL-12p35*, *IL-12p40*, *IL-23p19* and *IL-15* cytokine genes produced by different DC subsets. Since expression of mRNA may not correlate with their respective protein expression, quantitation of protein levels would be a more reliable method. Since it remains a technical challenge to quantitate cytokine production from a relatively small number of cells (DCs), the heavy reliance on mRNA quantitation posed a limitation to our study.

Conclusion

In maintaining immune homeostasis of the gut environment, two subsets of CD103⁺ DCs were found to have functionally distinct roles in mediating control of intestinal inflammation. Here we showed for the first time, both CD103⁺ DC subsets differentially regulate the outcome of colonic inflammation, through controlling epithelial barrier defense mechanisms. The up-regulation of anti-inflammatory IDO1, IL-18BP, mucins and AMPs in IECs during early DSS treatment highlights the anti-inflammatory and protective nature of CD103⁺CD11b⁻ DC subset. Whereas maintenance of IL-22BP expression in IECs mediated by CD103⁺CD11b⁺ DCs, as well as being the major source of IL-23, points to a pro-inflammatory role of this DC subset. Hence balancing the two opposing ‘forces’ with regards to controlling inflammation, is critical to ensure that pathogenic inflammatory conditions do not develop.

Figure 4.1. Schematic diagram summarizing the consequences of depleting $CD103^+CD11b^-$ and $CD103^+CD11b^+$ DCs during DSS-induced inflammation.

(A) Unaltered ratio of $CD103^+CD11b^-$ and $CD103^+CD11b^+$ colonic DCs resulted in mild inflammation. (B) Depletion of $CD103^+CD11b^-$ colonic DCs results in decreased IL-12-dependent IFN- γ production by $CD8^+$ tissue resident memory T cells leading to downregulation of IDO1, IL-18BP and MHCII expression in IECs. In addition, depletion of $CD103^+CD11b^-$ colonic DCs also lead to decreased production of AMPs by IECs and MUC2 secretion by goblet cells resulting in increased susceptibility to aggravated colitis. (C) Depletion of $CD103^+CD11b^+$ colonic DCs results in downregulation of IL-22BP by IECs which increases the local bioavailability of IL-22. High IL-22 activity promotes dramatic upregulation of AMPs in IECs and also supports regeneration of IECs leading to resistance to DSS-induced damage.

5. FUTURE WORK

We have demonstrated in a DSS-induced model of colonic inflammation that depletion of CD103⁺ CD11b⁻ DCs in Clec9A-DTR results in exacerbated colitis. Depletion of this DC subset in colonic LP resulted in compromised production of IL-12-dependent IFN- γ production by CD8⁺ IELs and CD4⁺ and CD8⁺ LP T cells resulting in reduced expression of IFN- γ -induced genes in IECs such as *Ido1*, *Il-18bp* and MHCII-related genes which are critical in controlling intestinal inflammation. However, we have not demonstrated the effect of this depletion on other IEL populations such as the IL-12 and IL-15 responsive IFN- γ -producing type 1 innate lymphoid cells, which has been demonstrated to contribute significantly to IFN- γ -mediated defense [53]. In addition, depletion of CD103⁺CD11b⁻ DCs results in compromised production of AMPs namely REG3 β , REG3 γ and S100A8/9 through mechanisms yet to be elucidated. The reduction of these AMPs contribute to a compromised epithelial barrier which may result in increased translocation of commensals to the mesenteric lymph nodes. Hence more work has to be done to elucidate the underlying mechanisms used by CD103⁺CD11b⁻ DCs to support IEC production of AMPs and the possibility of the compromised epithelial barrier function leading to increased commensal bacteria translocation to the mesenteric lymph nodes.

While we have demonstrated that depletion of CD103⁺CD11b⁺ colonic DCs protected mice from DSS-induced colitis through depletion of IEC-expressed IL-22BP, we have not established a cause-effect relationship of IL-22BP depletion with decreased susceptibility to DSS-induced colitis. In addition, we only speculated that a depletion of IL-22BP expression in IECs led to the dramatic increase in AMP production by IECs which protects mice from DSS-induced colitis. Hence more work has to be done to validate the potency of IL-22BP in regulating the outcome of colonic inflammation.

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