

**The characterisation of intestinal dendritic
cells and the control of immune responses
towards the microbiota**

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Abstract:

Dendritic cells (DCs) are regulators of the immune response and are thought to be critical in maintaining tolerance towards the intestinal microbiota. Recent data have identified distinct subsets of DCs with specific functional properties. The objective of this thesis was to further define CD103⁺ and CX3CR1⁺ DCs in the intestine and to determine how DCs and regulatory T (Treg) cell responses are influenced by the microbiota.

Using multicolour flow cytometry, we identified two CD103⁺ DC subsets with differential aldehyde dehydrogenase (ALDH) activity and two populations of CX3CR1⁺ cells. In the mesenteric lymph node CD103⁺ALDH⁺ DCs were highly mature (CD86^{hi}, MHCII^{hi}), likely migratory (CCR7⁺) and enhanced Treg cell induction compared with ALDH⁻ DCs. CX3CR1^{int} cells accumulated during bacterially-induced colitis suggesting a pro-inflammatory role whereas CX3CR1^{hi} cells were associated with the production of the anti-inflammatory cytokine IL-10 during homeostasis.

We also assessed the generation of CD103⁺ DCs from bone marrow progenitors. Although only small proportions of CD103⁺ DCs were detected in culture with FLT3L or GM-CSF alone, the combination of FLT3L and GM-CSF induced CD103⁺ DCs with a phenotype similar to those found in the small intestine. Using this system we showed that TLR ligands and retinoic acid induce ALDH enzyme activity *in vitro*.

In order to assess how DCs and Treg cells respond to changes in the microbiota we employed broad-spectrum antibiotic treatment to deplete endogenous bacteria and also analyzed the impact of colonization with the model organism *Helicobacter hepaticus*. Interestingly, we did not detect alterations in the proportions of different DC subsets following antibiotic treatment or *H. hepaticus* infection. However, using a novel FoxP3^{huCD2}-IL-10^{GFP} reporter mouse, we found that IL-10 production by Treg cells was ablated following antibiotic treatment and significantly elevated following *H. hepaticus* infection. Preliminary investigation of the mechanism underlying this effect suggests a role for IL-27. In summary, this thesis provides further detail on the phenotype of intestinal DCs and shows that Treg cell IL-10 production is sensitive to the composition of the microbiota.

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List of Abbreviations

<i>ADH</i> , alcohol dehydrogenase	<i>MDP</i> , muramyl dipeptide
<i>ALDH</i> , aldehyde dehydrogenase	<i>MLN</i> , mesenteric lymph node
<i>ASF</i> , altered Schaedler's flora	<i>MyD88</i> , myeloid differentiation primary-response protein 88
<i>Batf3</i> , basic leucine zipper transcription factor, ATF-like 3	<i>NK cell</i> , natural killer cell
<i>BMDC</i> , bone marrow-derived dendritic cell	<i>NKT cell</i> , natural killer T cell
<i>cDC</i> , conventional dendritic cell	<i>NLR</i> , nod-like receptor
<i>CLR</i> , C-type lectin receptor	<i>nTreg</i> , natural regulatory T cell
<i>CTLA-4</i> , cytotoxic T-lymphocyte antigen 4	<i>OVA</i> , ovalbumin
<i>DAMP</i> , damage-associated molecular pattern	<i>PAMP</i> , pathogen-associated molecular pattern
<i>DC</i> , dendritic cell	<i>pDC</i> , plasmacytoid dendritic cell
<i>DTR</i> , diphtheria toxin receptor	<i>PRR</i> , pattern recognition receptor
<i>FCS</i> , fetal calf serum	<i>NKT</i> , Natural Killer T Cell
<i>FLT3L</i> , Fms-related tyrosine kinase 3 ligand	<i>PSA</i> , polysaccharide A
<i>FoxP3</i> , forkhead box protein 3	<i>RA</i> , retinoic acid
<i>GALT</i> , gut-associated lymphoid tissue	<i>RAG</i> , recombinase activating gene
<i>GATA-3</i> , GATA binding protein 3	<i>RALDH</i> , retinaldehyde dehydrogenase
<i>GFP</i> , green fluorescent protein	<i>RNA</i> , ribonucleic acid
<i>GM-CSF</i> , granulocyte-macrophage colony-stimulating factor	<i>SED</i> , sub-epithelial dome
<i>H. hepaticus</i> , <i>Helicobacter hepaticus</i>	<i>SFB</i> , segmented filamentous bacteria
<i>IBD</i> , inflammatory bowel disease	<i>SPF</i> , specific pathogen free
<i>IEC</i> , intestinal epithelial cell	<i>STAT</i> , signal transducer and activator of transcription
<i>IFN</i> , interferon	<i>T-bet</i> , T-box transcription factor
<i>IL</i> , interleukin	<i>Tconv</i> , conventional (FoxP3-IL-10-) CD4+ T cells
<i>ILC</i> , innate lymphoid cell	<i>TCR</i> , T cell receptor
<i>ILF</i> , isolated lymphoid follicle	<i>TFH</i> , T follicular helper cell
<i>iLN</i> , inguinal lymph node	<i>TGF-β</i> , transforming growth factor β
<i>IRF</i> , interferon regulatory factor	<i>Th</i> , T-helper
<i>iTreg</i> , induced regulatory T cell	<i>TiP DC</i> , TNF- α , iNOS producing dendritic cell
<i>LAP</i> , latency-associated peptide	<i>TLR</i> , toll-like receptor
<i>LC</i> , Langerhans cell	<i>TNF-α</i> , tumour necrosis factor α
<i>LPL</i> , lamina propria leukocytes	<i>Tr1 cell</i> , type 1 T regulatory cell
<i>LPS</i> , lipopolysaccharide	<i>Treg</i> , regulatory T cell
<i>LTi cell</i> , Lymphoid tissue-inducer cell	<i>TRIF</i> , Tir-domain-containing-adaptor protein inducing IFN- β
<i>MAMP</i> , microbe-associated molecular pattern	<i>TSLP</i> , thymic stromal lymphopoeitin
<i>M-cells</i> , microfold cells	<i>UC</i> , ulcerative colitis
<i>M-CSF</i> , macrophage colony-stimulating factor	

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Chapter 1

General Introduction

1.1 Overview

This thesis covers aspects of intestinal dendritic cell (DC) and regulatory T (Treg) cell biology which contribute towards maintaining a mutually beneficial (mutualistic) relationship with resident intestinal bacteria (henceforth referred to as the 'microbiota'). The importance of this mutualistic relationship is clear from the chronic immune-mediated pathology that develops as a consequence of its breakdown [1]. In the intestine this pathology manifests as Inflammatory Bowel Disease (IBD) [2]. Particularly relevant points are raised in short introductions preceding the presentation of data. In addition, recently published data that has emerged within the last two years and that has direct relevance to our studies is discussed in detail in sections at the end of the relevant chapters. However, in order to place this work into a wider context and to explain our approach, it is important to describe more generally the role DCs play in influencing immune responses and especially how they act within the intestinal immune system to orchestrate mutualism.

1.2 Dendritic cells

1.2.1 Dendritic cells: General overview

DCs were first defined in the spleen and peripheral lymph nodes based on their morphological appearance in 1973 [3], although similar cells had been identified in the skin by Paul Langheran over 100 years earlier [4-6]. Nomenclature in immunology can often be misleading, however the term "dendritic", drawn from the

“fine cell processes” which protrude from their surface, rather neatly hints towards DC function, which is to interact with other cells of the immune system and to initiate adaptive immune responses via the presentation of antigen [7].

The importance of DCs in initiating immune responses is best demonstrated by the effect of DC-depletion *in vivo*. In these models the diphtheria toxin receptor (DTR) is expressed under the control of the promoter for CD11c, an integrin highly expressed by murine DCs. Injection of these mice with diphtheria toxin therefore results in the targeted depletion of the DC population [8-10]. DC-depletion prevents the initiation of antigen-specific CD4 and CD8 T cell responses *in vivo* and the acquisition of T cell effector functions, thus indicating that DCs have a non-redundant role in the initiation of adaptive immune responses [9, 11, 12]. Importantly, this is also true for the development of antigen-specific T cell responses in the gut associated lymphoid tissue (GALT) following oral vaccination [13]. Additional requirements for DCs in maintaining NK cell numbers via IL-15 secretion [10] and controlling homeostatic Treg cell numbers [14] were also revealed in these models. Thus, although DCs form a relatively rare cell population *in vivo* (~3% of total splenocytes), they provide a crucial bridge between innate and adaptive immunity and can modulate immune responses in a remarkably potent manner.

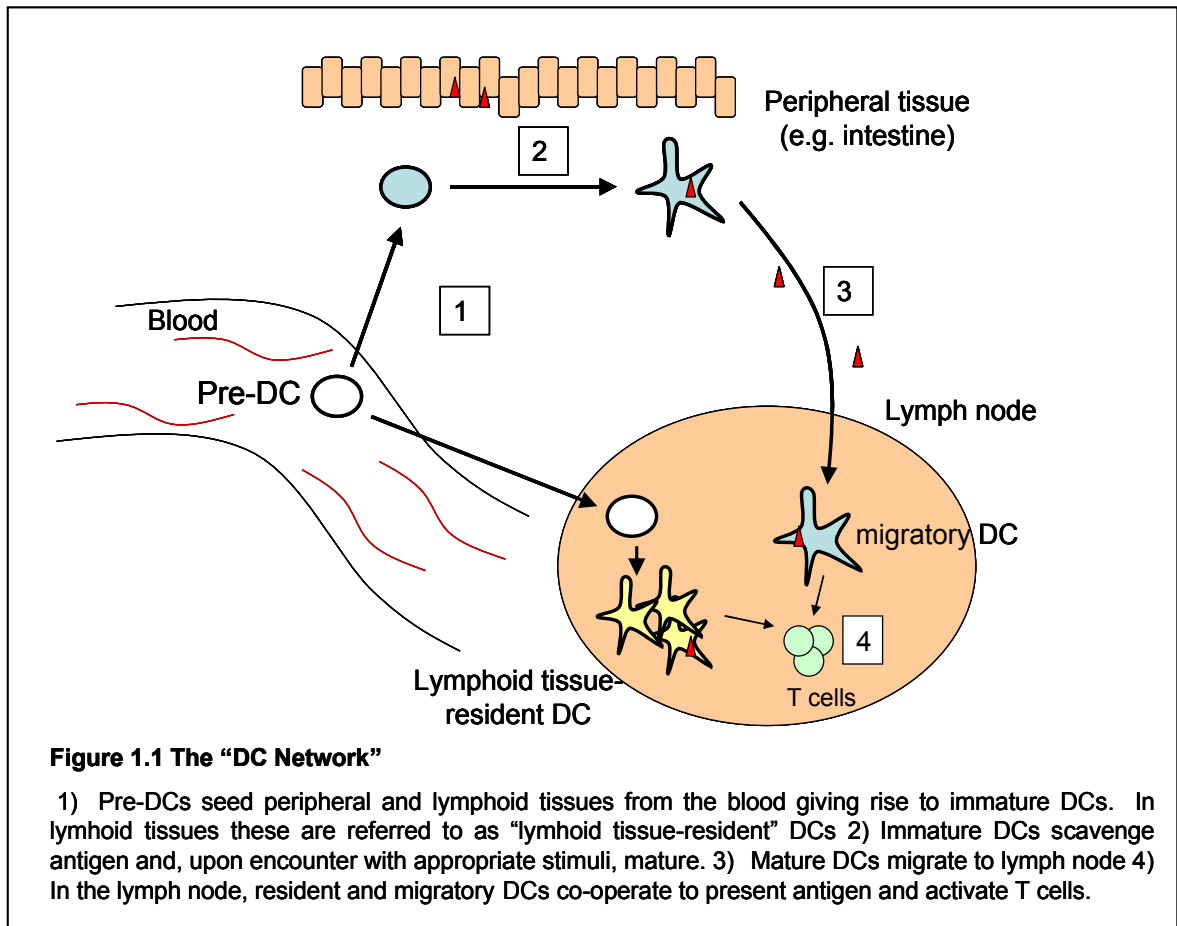
The simplicity of the statement “DCs initiate adaptive immune responses” belies the complex range of processes that this actually entails. Firstly, antigen must be taken-up (*antigen capture*) often from diverse locations *in vivo*. Secondly, this antigen must be transported to lymphoid tissue and to areas where lymphocytes are clustered (*antigen carriage*) [15]. Thirdly, antigen must be presented (*antigen presentation*) in a context capable of inducing a proliferative response and finally,

this nascent response must be directed towards an outcome suitable for the type and level of threat (*T-helper cell polarisation*) [16].

Therefore, it is incorrect to think of DCs as a single mono-functional entity. Rather they form a constitutively active “sentinel network”, which is continually engaged in the cycle of antigen capture, carriage and presentation (**see figure 1.1**). Furthermore, in order to fulfil the diverse requirements that this cycle demands, DCs undergo remarkable phenotypic maturation so that at any one time DCs form a functionally heterogeneous cell population. An overview of the fundamentals of DC biology, including their ontogeny, separation into distinct subsets and the functional changes which accompany maturation is provided below and considered in more detail in the context of the intestinal immune system later on. For further detail on conventional DC biology that is beyond the scope of this introduction the reader is referred to [7].

1.2.2 Dendritic cells: DC ontogeny

DCs can be found in both lymphoid tissue, where the presentation of antigen occurs, and in non-lymphoid peripheral tissues, where they are thought to act as sentinels for the capture of antigen and the detection of invading pathogens [17-19]. It is now apparent that both lymphoid and non-lymphoid tissue DCs derive from bone marrow-derived precursors, which circulate in the blood and differentiate into DCs within the respective tissues [20-25] (**see figure 1.1**). The nature of these precursors and the factors that control their differentiation into DCs has been the subject of intense research over the past decade. Broadly speaking DCs can be generated from circulating pre-DCs in a FLT3L-dependent fashion [23, 26-28] or from monocytes in a GM-CSF-dependent fashion [29].

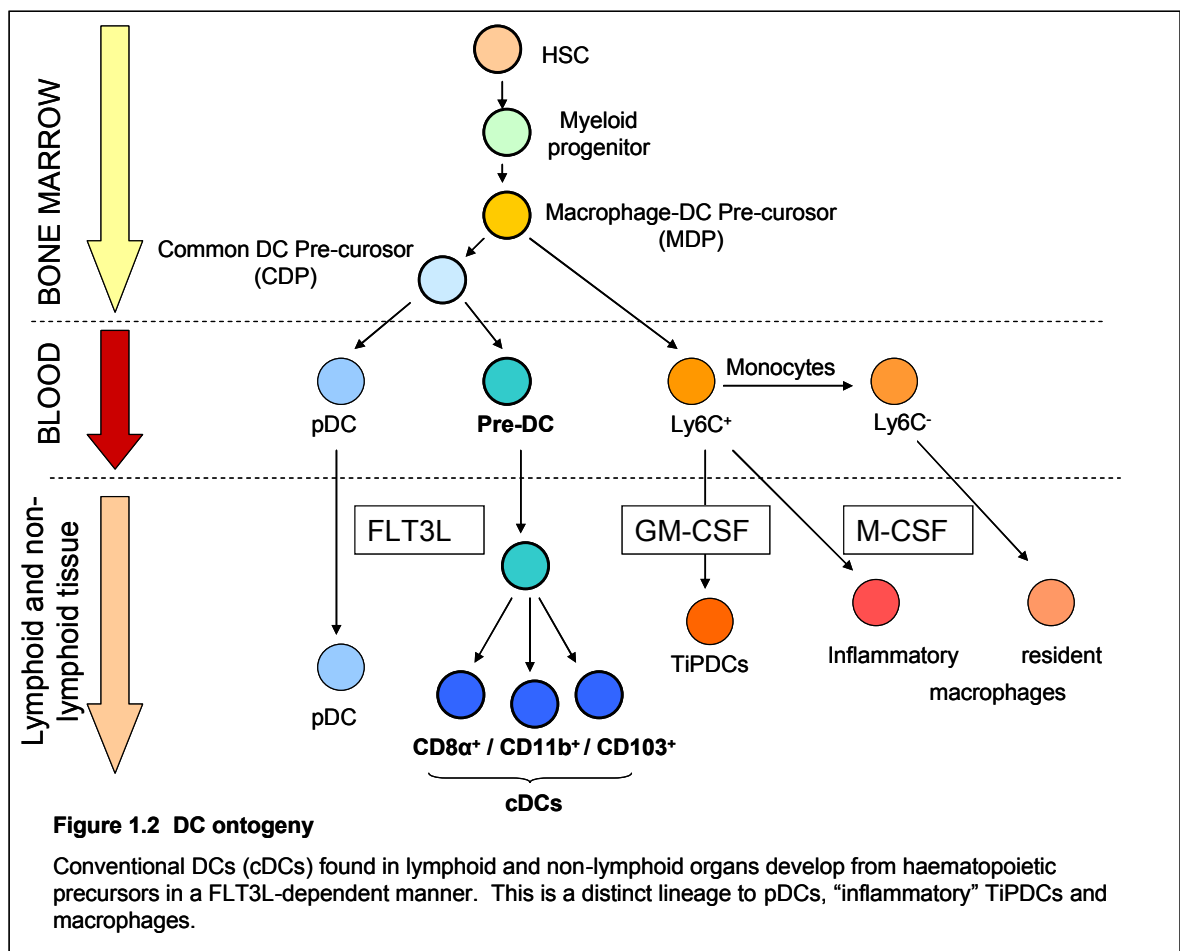


It is apparent that the pre-DC, FLT3L-driven lineage predominates during homeostasis, giving rise to nearly all conventional DCs in the lymphoid tissue and the majority of DCs in non-lymphoid tissue [26, 28]. It is also apparent that lymphotoxin- β can sustain proliferation of splenic CD11b⁺ DCs suggesting that this may supplement the role of FLT3L in maintaining this DC population [30]. It is possible that some sub-populations of DCs in non-lymphoid tissues and in peripheral lymph nodes may arise from monocytes during homeostasis in a GM-CSF or even M-CSF dependent manner [25, 31-34]. However, these cells often appear to fill specialised niches, e.g. CX3CR1⁺ DCs in the intestine, and are less-likely to fulfil all the archetypal DC functions described above [35].

During inflammation or in response to infection the DC network is supplemented by the infiltration of Gr1^{hi} monocytes whose differentiation into DCs is GM-CSF-

mediated [36-39]. These populations of monocyte-derived DCs can clearly present antigen to naive T cells in the manner of conventional DCs, however there is little evidence that they become migratory and travel to lymph nodes in order to do so [39]. Rather they appear to fulfil more of an “innate” function, producing TNF- α and expressing iNOS, to resist pathogens at the site of infection [38]. Such DCs have been described as TNF- α -iNOS producing DCs (TiP-DCs) to take this phenotype into account.

A current overview of DC development is provided in **figure 1.2**.



1.2.3 Dendritic cells: Sets and subsets

Apart from the distinction between monocyte, GM-CSF-derived DCs and pre-DC, FLT3L-derived DCs it is possible to draw further distinctions between sets and subsets of DCs. The first major distinction can be drawn between conventional DCs (cDCs), those described by Steinman *et al*, and plasmacytoid DCs (pDCs). pDCs and cDCs arise from a common precursor (Common DC Progenitor: CDP) [27, 40] in a FLT3L-dependent fashion [28]. However, unlike cDCs, pDCs remain in something of a precursor state and circulate in the blood. Not unlike monocytes, pDCs are relatively small, round cells and require substantial further differentiation to achieve a “dendritic” form. pDCs, rather than specialising in antigen capture and presentation, appear to offer more by way of innate function, in particular releasing large quantities of type I interferons (IFNs) in response to viral or microbial stimuli [41]. Recently, further sub-populations of pDCs have been described with distinct functional properties [42-44]. However, the precise detail and ontogeny of these sub-populations remains unclear.

After commitment towards cDCs, DC precursors can further differentiate into distinct subsets all of which are FLT3L-dependent. In lymphoid tissues, cDC subsets have been described based primarily on the markers CD8 α , CD11b and CD4 [45, 46]. CD8 α^+ CD11b $^-$ CD4 $^-$ are enriched within the T cell zones [47] and are more efficiently able to cross-present to and cross-tolerise CD8 $^+$ T cells [48, 49]. By contrast, CD8 α^- CD11b $^+$ DCs (which can be further separated in CD4 $^+$ and CD4 $^-$ subsets) are thought to reside primarily in the marginal zone [47] where they are better able to scavenge antigen [50]. Importantly the development of CD8 α^+ CD11b $^-$ DCs and CD8 α^- CD11b $^+$ DCs has been associated with distinct transcription factors suggesting distinct programs of differentiation for each DC

subset rather than merely acquisition of additional surface markers [18]. In particular, CD8 α^+ CD11b $^-$ DCs are highly dependent upon Batf3 and IRF-8 expression for their development [51-53], whereas CD8 α^- CD11b $^+$ DCs are more reliant on RelB, IRF-2 and IRF-4 [54-56]. Human IRF8-deficiency has also been associated with an absence of CD11c $^+$ and CD123 $^+$ pDCs resulting in an inability to contain *Mycobacterium bovis* infection following Bacille Calmette-Guerin (BCG) vaccination [57].

Recently, it has become evident that distinct DC subsets also exist among the DC populations in non-lymphoid tissues [18, 58]. In particular, DCs expressing the α_E -integrin, CD103 [59, 60], or the fractalkine receptor, CX3CR1 [61, 62], have been suggested to represent distinct DC subsets enriched at mucosal surfaces, such as the intestine. In addition, Langerhans Cells (LCs) which reside in the epidermis and depend on M-CSF and TGF- β for their differentiation have a unique phenotype. In particular, LC numbers are maintained by self-renewal rather than continual recruitment of blood borne precursors [63]. How these non-lymphoid tissue DC populations relate to each other or to DCs residing in lymphoid tissues is an active area of research.

Finally, after engaging antigen or pathogen in the periphery, DCs migrate to lymph nodes in order to affect antigen presentation. This process of migration is constitutive [64, 65], but substantially increases following infection or the application of inflammatory stimuli [66-68]. Therefore lymph node DCs can also be separated into distinct sub-populations based on whether they differentiate and reside in the lymphoid tissue itself (Lymphoid tissue-resident DCs) or whether they have migrated in from the periphery (Migratory DCs). A recent elegant study has

indicated that only a small proportion of DCs in the lymph node are migratory under homeostatic conditions [69].

1.2.4 Antigen capture: Focus on the intestine

Regardless of whether a DC resides in lymphoid tissue, such as the spleen, or in the periphery the primary function remains the same: capture antigen and present antigen to T cells. Newly differentiated “immature” DCs are intrinsically specialised to affect antigen capture via phagocytosis, macropinocytosis and receptor-mediated endocytosis [7]. In the intestine there are particular mechanisms in place which allow DCs to take up antigen from the lumen. As we shall see such low level of exposure to antigenic stimulation is important in the DC mediated control of host defence and tolerance. Specialised microfold cells (M-cells), located in the follicular associated epithelium, transport antigen across the epithelium for uptake by DCs in the sub-epithelial dome (SED) of Peyer’s Patches [70]. Away from the Peyer’s Patches, opsonised antigen can be transported across the epithelium via Fc receptors, such as neonatal Fc receptor [71]. Finally, immunofluorescence studies have suggested that DCs are able to extend dendrites through the epithelial layer to directly sample luminal antigen [62, 72, 73].

In addition to capturing extracellular antigen derived from bacteria, DCs also take up both necrotic and apoptotic cells via specific endocytic pathways [74, 75]. In this way DCs acquire intracellular antigen resulting from infection, which is important to generate adaptive immunity, and apoptotic cell-derived “self” antigen, which is used to promote tolerance.

1.2.5 DC maturation

Following antigen capture and in the presence of appropriate stimuli, “DC maturation” results in a change of DC phenotype and the acquisition of a characteristic “dendritic” structure. In particular, DCs lose responsiveness to peripheral tissue-associated chemokine gradients, such as CCL20 which signals through CCR6, and instead gain responsiveness to CCL19/CCL21. These latter chemokines signal via CCR7 and induce DC migration along lymphatic vessels towards the lymph node and into the T cell zones [76, 77]. Few DCs can be found exiting lymph nodes in efferent lymph, although it has been suggested that on rare occasions DCs can re-circulate to the bone marrow [78] or even the thymus [79, 80]. Therefore most migratory DCs presumably die in the lymph node following the presentation of antigen [15]. This has the advantage of allowing antigen to be recycled via the uptake of apoptotic migratory DCs by lymphoid-tissue resident DC populations.

Also accompanying DC maturation are changes which enable the DC to give the “three signals” required for T cell activation. Firstly, up-regulation of peptide-bound Major Histocompatibility Complex Class II (MHCII) expression on the cell surface enabling presentation of antigen. Secondly, co-stimulatory molecules such as CD80, CD86 and CD40 are expressed on the cell surface of mature DCs enabling T cell co-stimulation to take place. Thirdly, DC maturation is accompanied by the production of cytokines that mediate a wide range of processes and, importantly for the development of an adaptive response, direct complete differentiation of effector T cells [17].

1.2.5.1 Pattern recognition receptors (PRRs): TLRs/NLRs/CLRs

DC maturation is not driven by the uptake of antigen *per se* but rather by other signals present in the local environment which give the process of antigen uptake a degree of context. These signals can include bacterial products such as Lipopolysaccharide (LPS), (so-called pathogen- or microbe-associated molecular patterns; *PAMPs/MAMPs*), products released from necrotic cells (so-called damage-associated molecular patterns; *DAMPs*) [81], pro-inflammatory cytokines, such as TNF- α (so-called “*bystander*” activation), and signals from activated T cells, such as CD40-ligation.

The recognition of PAMPs, MAMPs and DAMPs is mediated by a collection of different pattern recognition receptors (PRRs), so named because of their ability to bind specific molecular patterns found in a range of ligands. PRRs encompass a range of different receptor families, most notably Toll-like receptors (TLRs), Nod-like receptors (NLRs) and C-type lectin receptors (CLRs) [82, 83]. Different families and family members have specificity for different molecular patterns thus enabling “danger” from a wide range of sources to be recognised. For example, TLR4 recognises lipopolysaccharide (LPS) a major constituent of gram negative bacteria. NOD2 recognises muramyl dipeptide (MDP), often indicative of gram positive bacteria. TLR3 and TLR7 recognise viral RNA and zymosan, a constituent of the fungal cell wall, is recognised by Dectin-1, TLR2 and TLR6. PRRs are also compartmentalised within the cell which has clear implications for microbial recognition. TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface, although these can be internalised following ligand binding, whereas TLR3, TLR7, TLR8 and TLR9 are constitutively localised to endosomes. Thus TLR2, TLR4 and TLR5 largely detect extracellular bacteria or fungi whereas TLR3, TLR7, TLR8 and

TLR9 all detecting nucleic acids require some process of phagocytosis or endocytosis.

TLR signalling, which brings about DC maturation, is notoriously complicated with ligation and receptor dimerisation recruiting numerous different adaptor and signalling proteins [84, 85]. In this way, ligation of specific TLRs is translated into a transcriptional response, often via NF- κ B, and ultimately a distinct mature DC phenotype [86]. The intricacies of TLR signalling are far beyond the scope of this thesis, but it is worth highlighting two fundamental adaptor proteins which are relevant to the data presented and subsequent discussion. MyD88 (myeloid differentiation primary-response protein 88) is an essential adaptor for the signalling of all TLRs except for TLR3 and for some responses of TLR4, and also for IL-1R and IL-18R-signalling [87]. Therefore, mice deficient in MyD88 are unresponsive to many TLR ligands and in particular fail to produce inflammatory cytokines in response to infection [87]. An alternative, MyD88-independent pathway acts downstream of TLR3 and TLR4 [88]. This pathway utilises the adaptor TRIF (Tir-domain-containing-adaptor protein inducing IFN- β) [89, 90] and as the name suggest is specialised for the production of type I IFNs, in particular IFN- β , but not the majority of inflammatory cytokines, such as IL-6, IL-1 β and TNF- α [88]. Importantly, IFN- β can have autocrine signalling effects in some cell types (e.g. macrophages and DCs) to induce or enhance further gene transcription (e.g. IL-27).

As mentioned above, DCs mature and migrate constitutively, even in the absence of infection or inflammation. In this setting, where PRR signals are presumably limited, it remains unclear precisely which signals induce DC maturation.

Importantly, unlike the uptake of necrotic cells, the uptake of apoptotic cells which are a major source of 'self' antigen is not sufficient to induce DC maturation [91].

1.2.6 Dendritic cells: Antigen presentation and the control of T cell activation

In order to efficiently activate T cells and so initiate immune responses, DCs must provide three signals [7]. The first signal is antigen presentation on MHC-I (to CD8⁺ T cells) or MHC-II molecules (to CD4⁺ T cells). The second is co-stimulation primarily via the ligation of CD28 on the T cell with CD80 or CD86 on DCs. This elicits cytokine secretion in particular IL-2 and the expression of anti-apoptotic molecules, which support T cell proliferation and survival [92]. Notably, immature DCs, which do not express co-stimulatory molecules, are unable to provide this second signal [93] and so immature DCs do not initiate complete immune responses. Instead antigen-presentation by immature DCs results in transient T cell proliferation and ultimately deletion or anergy. In this way, antigen taken up in the absence of maturation signals, such as following engulfment of apoptotic cells, is effectively tolerated [94]. Furthermore, elegant work by Blander *et al* indicated that T cell activation will not occur unless the maturation stimuli are taken up into the same phagosome as antigen [95]. Thus, subsequent maturation of DCs which have taken up self antigen is not sufficient to induce effective antigen presentation. This requirement for maturation in the induction of immune responses as opposed to tolerance represents the first major way in which DCs exert control over the adaptive immune system.

Previous to the work of Blander *et al*, Sporri *et al* had observed a similar phenomenon *in vivo* [96]. This study showed that bystander activation of DCs by inflammatory cytokines alone was insufficient for the induction of complete T cell effector responses. Rather antigen-presenting DCs must be able to respond

directly to the exogenous TLR agonist. Investigation of the underlying mechanism showed that DCs must be able to produce cytokines (in this case IL-12) which provide “signal three” to T cells driving their differentiation and polarisation into effector T cell subsets [97].

1.2.7 T cell restimulation in peripheral tissues

Emerging evidence suggests that activated CD4⁺ T cells that have homed to peripheral tissues can be further modulated by DC-dependent [98-100] or DC-independent mechanisms [101, 102]. For example re-stimulation by DCs in the skin is required to licence full effector function even after the immune response is initiated [98]. However, the requirement of intestinal DCs to act in the same way is unknown. DC-independent mechanisms suggest that CD4⁺ T cells can act in a more innate manner in peripheral tissue. For example, TLR2 activation on Th17 cells plays an important role in driving inflammation in a model of central nervous system immune pathology [101]. Conversely, TLR2 activation on Treg cells can enhance tolerance in the intestine [102] and elsewhere [103]. Given the ability of several cell populations to express MHCII it is conceivable that many cell types are capable of influencing T cell function by re-stimulation in the periphery, including stromal cells, myeloid cells and B cells.

1.3 Dendritic cells and CD4⁺ T cells: Directing the immune response

1.3.1 An overview of CD4⁺ T-helper cell responses

Following antigen presentation CD4⁺ T cells undergo clonal expansion and differentiate into distinct T-helper (Th) subsets with characteristic cytokine profiles and effector functions [104]. In a slightly simplistic overview: Th1 cells produce IFN- γ and assist cell mediated immunity against intracellular pathogens, Th2 cells

produce IL-4 and IL-13 and assist the humoral immune response and Th17 cells produce IL-17 and IL-22 and promote immunity against extracellular pathogens including fungi. Additionally, Treg cells are often described as a T-helper cell subset producing IL-10, IL-35 and TGF- β and affecting tolerance via immune suppression. In actual fact, Treg cells can be split into two distinct populations of their own. “Natural” Treg (nTreg) cells arise in the thymus by positive selection whereas “induced” Treg (iTreg) cells arise in the periphery following antigen presentation [105]. Therefore, iTreg cells are similar to the induced T-helper subsets which arise following naive T cell activation, whereas the generation of nTreg cells has slightly different requirements (this is discussed further below) [106].

CD4⁺ T cell differentiation is driven by specific cytokine signals, the activation of distinct STAT proteins, expression of canonical transcription factors and epigenetic remodelling [107]. Th1 cell differentiation is driven by IL-12 signalling and is dependent on STAT4 activation (with an additional role for STAT1) and the transcription factor, T-bet [108]. Th2 cell differentiation is driven by IL-4 signalling [109, 110] and is dependent on STAT6 activation [111-113] and expression of the transcription factor GATA-3 [114, 115]. Th17 cell differentiation is driven by co-operative TGF- β and IL-6 signalling in the mouse [116-118] (human Th17 cell differentiation has slightly different requirements [119]) and is dependent on STAT3 activation and the transcription factor ROR γ t [120-122]. Interestingly, TGF- β signalling in the absence of IL-6 drives iTreg cell differentiation [123] dependent upon expression of the transcription factor, FoxP3 [124-126]. Thus, Th17 and iTreg cell subsets are closely related yet divergent.

Although the above Th subsets are well established the identity of other proposed Th subsets is more controversial. These include Th9 cells [127], producing IL-9, T regulatory 1 (Tr1) cells [128], producing IL-10, and T follicular helper (T_{FH}) cells producing IL-21 and expressing CXCR5 which localises them to germinal centres [129]. The controversy centres on whether these cells really form distinct lineages or whether they simply represent acquired functions of the aforementioned Th populations. T_{FH} cells depend on expression of the transcription factor Bcl-6 [130, 131]. However canonical transcription factors have not been described for either Th9 or Tr1 cells.

More generally the concept of Th cell subsets being immutable even in counter-polarising conditions has been challenged. Evidence of dual expression of canonical cytokines, canonical transcription factors [132] and elegant “fate-mapping” studies tracking T-helper cell phenotype over time indicate considerable plasticity between subsets [133, 134]. This has particularly been evident in the intestine where FoxP3⁺ CD4⁺ T cells have been suggested to convert to Th17 cells [135] and to T_{FH} cells [136]. Furthermore, this plasticity may be of critical functional importance. For example, recent evidence indicates that FoxP3⁺ Treg cells require expression of T-bet to efficiently suppress Th1 cell-mediated inflammation [132] and similar cross-transcriptional requirements have been observed for the control of Th2 cell-mediated pathology [137].

Nevertheless the importance of directing “appropriate” T-helper cell differentiation to target “appropriate” immune responses is well established. In a seminal experiment Blumberg *et al* showed that the initiation of Th2 cell response rather than Th1 cell response in Balb/c mice following *Leishmania* infection led to failure to cope with the intracellular infection and a catastrophic outcome for the host

[138]. Similarly, one can imagine that activation of a Th1, Th2 or Th17 cell response when in fact Treg cell-mediated tolerance is desired can have auto-inflammatory consequences. Therefore, directing T-helper cell differentiation and thus immune response to different stimuli is paramount.

1.3.2 The control of CD4⁺ T cell differentiation by DCs

As well as regulating the initiation of immune responses, DCs influence the type of response that is generated by directing Th cell differentiation accordingly. This is achieved via the production of the Th cell polarising cytokines and complemented by co-stimulation. Evidence suggests DCs are specialised to affect differential Th cell responses in two main ways. Firstly, the intrinsic nature of distinct DC subsets and secondly, differential DC maturation resulting from the integration of local environmental signals [16, 139].

The intrinsic ability of lymphoid tissue CD8 α^+ CD11b $^-$ and CD8 α^- CD11b $^+$ DC subsets to promote different CD4⁺ T cell responses has been well documented. The adoptive transfer of antigen loaded CD8 α^+ CD11b $^-$ DCs induced primarily Th1 cell differentiation via the production of IL-12. By contrast adoptive transfer or expansion of CD8 α^- CD11b $^+$ DC induces a primarily Th2 cell response although a definitive mechanism for this remains unclear [140, 141]. Given that CD8 α^+ CD11b $^-$ DCs are also preferentially able to cross-present antigen to activate CD8⁺ T cells this would seem to suggest that CD8 α^+ CD11b $^-$ DCs are specialised to promote immune responses to target intracellular pathogens. A similar dichotomy has been observed in human studies, although it is not clear quite how different subsets overlap between human and mouse [142].

It is clear that any intrinsic predisposition for specific Th cell induction can be modulated, and indeed over-ridden, by treatment of DCs with different cytokines, different microbial agents or by altering the DC: T cell ratio used for stimulation [143, 144]. Furthermore, detailed gene-expression analysis of DCs stimulated with *E-coli*, *Candida albicans* or influenza virus indicated that these different pathogens induced different maturation phenotypes [145]. Furthermore, different TLR agonists combine to specify a transcriptional signature which specialises the DC to induce a specific T-helper cell response [146]. For example, activation of DCs by fungal products, like curdlan, induces Th17-promoting cytokines such as IL-6 and IL-23, but not Th1 promoting cytokines such as IL-12. Therefore, stimulation of DCs by fungi gives rise to Th17 responses appropriate for most efficient clearance [147].

DC responses can also be tuned by additional products present in the local environment. These can include different cytokines, such as IFN- γ (which likely enhances Th1 induction) [144], TSLP (which enhances Th2 induction) [148] and IL-10 (which enhances Tr1 cell-induction) [149], and co-factors such as VitD3 [150] or retinoids [151]. The combined effect of different environmental factors on DC maturation is commonly referred to as “DC conditioning”. DC conditioning in the context of the intestinal immune system is discussed later.

1.3.3 The control of regulatory T cells by DCs

Given the importance of remaining tolerant to self antigen and, in the intestine, to microbial antigen much interest is focused on how DCs control Treg cell responses [152]. There is evidence that immature DCs promote iTreg cell induction *in vitro* [153] and *in vivo* [154, 155] consistent with the suggestion that strong co-stimulation is inhibitory for iTreg cell development [156]. However, it has

recently become apparent that mature DC populations can also be specialised for iTreg cell induction [157, 158]. Furthermore, recent studies have shown that DCs play a broad role controlling Treg cell numbers *in vivo*. Constitutive DC depletion by expression of the diphtheria toxin A subunit under control of CD11c promoter resulted in widespread autoimmunity driven by dysregulated T cell responses, suggesting a possible defect in immune regulation [159]. This lymphoproliferative phenotype was not observed in a similar constitutive depletion model suggesting that there may be an environmental trigger for this pathology [8, 160]. In other studies, transient depletion or expansion of DCs *in vivo* correlates with decreased or increased numbers of FoxP3⁺ Treg cells respectively, and this is dependent on MHCII expression [14, 161]. This is consistent with data suggesting DCs can support the proliferation of Treg cells *in vitro* [162]. Therefore, it is likely that DCs exert control over the initiation of immune responses and both Th cell differentiation and Treg cell function.

1.4 Overview of the intestinal immune system: The importance of maintaining mutualism

Mutualism describes a relationship between two species which is advantageous to both. In the intestine, the microbiota contribute to maturation of the immune system, to defence against opportunistic pathogens, to carbohydrate digestion, to angiogenesis and to peristalsis, whilst obviously gaining a niche rich in nutrients in which to reside [163]. This influence of the microbiota on a wide range of mammalian functions is demonstrated by the diverse array of transcriptional changes, encompassing components of all the processes listed above, which occur in the intestine following colonisation of germ-free mice (raised in sterile

conditions) with *Bacteroides thetaiotaomicron* [164]. Therefore, the host's relationship with the intestinal microbiota is considered to be mutualistic.

However, maintaining this mutualistic relationship is problematic for the intestinal immune system. Inflammatory immune responses against the microbiota underlie the pathogenesis of many mouse models of inflammatory bowel disease [165-168] and transgenic mice expressing TCRs specific for bacterial antigens can be induced to drive intestinal inflammation [169-171]. Conversely, the inherent permeability of the intestine makes it a vulnerable site for pathogen entry and even members of the microbiota, such as *Bacteroides fragilis*, can be opportunistically pathogenic in conditions of immune-deficiency [172]. Therefore the immune system must be able to mount a robust defence against intestinal bacteria whilst simultaneously, and seemingly paradoxically, tolerating its presence. Described here are the processes of host defence and tolerance in the intestine which resolve this paradox. Importantly, the microbiota is not a passive bystander in this system but actively modulates the immune response in ways that can both benefit and damage mutualism.

1.4.1 The intestinal immune architecture

The intestinal immune system consists of a substantial architecture of lymphoid tissue and a large number of dispersed immune cells which collectively act to manage immune responses locally without engaging a systemic immune response (**see figure 1.3**). Lymphoid structures such as aggregated lymphoid follicles (Peyer's patches) and isolated lymphoid follicles (ILFs) are present in the intestinal tissue itself. DCs migrate to draining lymph nodes, primarily the mesenteric lymph nodes (MLN), and T and B cell responses are targeted back towards the mucosa. These cells join a substantial network of innate and adaptive cells dispersed

throughout the lamina propria and the intestinal epithelial layer which provide localised effector functions [173]. DCs transport phagocytosed bacteria to the MLN, but do not penetrate further [174]. Therefore, the intestinal immune system has been described as a “firewall” enabling the systemic immune system to remain largely ignorant to the presence of intestinal bacteria.

It has been clear from some of the earliest studies on germ-free mice that intestinal bacteria are essential for the complete maturation of the immune system. There are reduced numbers of T and B cells in the intestinal lamina propria of germ-free mice culminating in an absence of T helper cells and IgA-producing plasma cells [175-177]. Additionally, there is a general failure to organise germinal centre formation in the Peyer’s Patches, lymph nodes and spleen of germ-free mice [178, 179]. All these defects are correctable by colonisation of germ-free mice with a conventional flora. Therefore, many of the systems required for prevent infection are instigated by the intestinal microbiota itself.

The inhibition of inflammatory responses (*tolerance*) whilst still resisting infection (*host defence*) may at first seem paradoxical. In fact, these two processes work together to maintain mutualism [172]. Host defence mechanisms prevent intestinal bacteria from penetrating beyond the intestinal lamina propria and so reduce the interaction between bacteria and immune cells. Simultaneously, mechanisms of tolerance balance host defence and limit the level of the inflammatory response to avoid the development of immune-mediated pathology. These processes are discussed below.

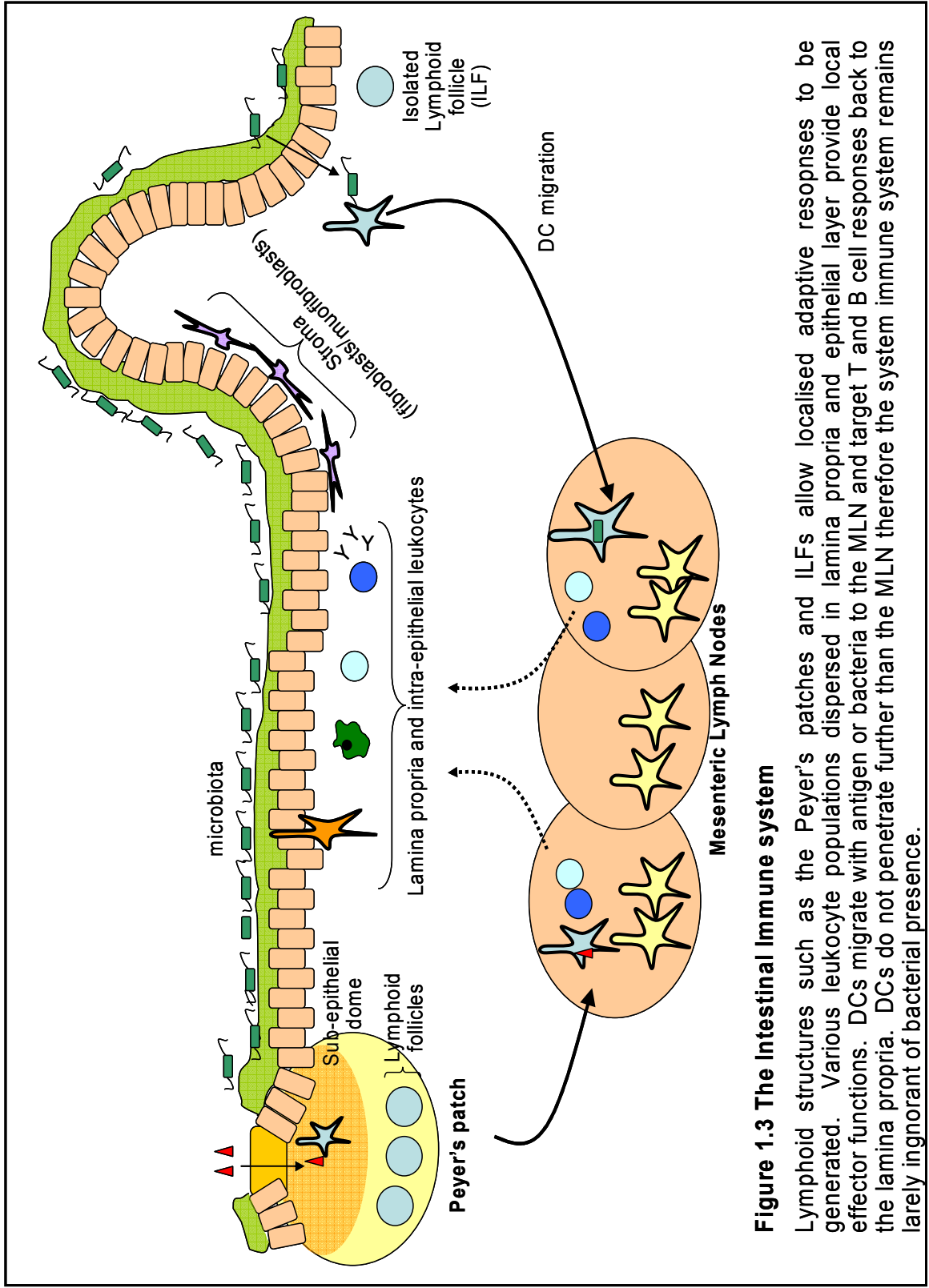


Figure 1.3 The Intestinal Immune system

Lymphoid structures such as the Peyer's patches and ILFs allow localised adaptive responses to be generated. Various leukocyte populations dispersed in lamina propria and epithelial layer provide local effector functions. DCs migrate with antigen or bacteria to the MLN and target T and B cell responses back to the lamina propria. DCs do not penetrate further than the MLN therefore the system immune system remains largely ignorant of bacterial presence.

1.4.2 The maintenance of mutualism: Host defence

At first glance all that seems to stand between us and infection by 10^{14} commensal bacteria is a single layer of intestinal epithelial cells. In fact, several host defence mechanisms exist to prevent the commensal flora from adhering to and potentially invading this epithelial cell layer. These host defence mechanisms include the maintenance of an antimicrobial barrier as well as both innate and adaptive immune responses involving phagocytosis, antibody (IgA) production and cytokine production. The efficiency of host defence is clear from the fact few bacteria can be cultured from the MLN or spleen of wild-type mice. By contrast bacteria can be readily cultured from these organs if mice are deficient in host defence pathways (e.g. MyD88^{-/-} or MyD88^{-/-}TRIF^{-/-} mice) [180, 181]. Furthermore, MyD88^{-/-}TRIF^{-/-} mice also show elevated systemic antibody responses against intestinal bacteria indicating that a break down in host defence can cause the initiation of immune responses against the microbiota [180]. The potential inflammatory consequences of this breakdown in ignorance are clearly demonstrated in animal models where defects in host defence pathways, such as bacterial sensing [182], mucin production [183], epithelial cell survival [184, 185], autophagy (important for the secretion of antimicrobial peptides) [186, 187] and inflammatory cytokine production (e.g. IL-18) [184][188, 189] cause spontaneous or exacerbated intestinal inflammation.

As one might expect if the trigger for inflammation is enhanced exposure to bacteria, many of these models are associated with changes in the microbiota [187, 190, 191]. For example, T-bet^{-/-}RAG^{-/-} develop colitis triggered by the outgrowth of *Klebsiella pneumoniae* and *Proteus mirabilis* strains [190]. Therefore, colitis can be transferred across strains via transfer of the microbiota either by

gavage of caecal contents or by the cross-fostering of pups. Conversely the removal of colitogenic bacteria by re-derivation of these strains into new facilities can result in a loss of colitis-phenotype (e.g. TLR5^{-/-} mice and T-bet^{-/-}RAG^{-/-} mice fail to develop spontaneous intestinal inflammation in our facility, K. Maloy, F. Powrie unpublished data). Interestingly, *Klebsiella pneumoniae* and *Proteus mirabilis* are not sufficient to cause colitis if transferred alone to germ-free mice [190] suggesting a complex interplay whereby the outgrowth of these bacteria causes a fundamental reorganisation of the bacterial community to the detriment of mutualism. Therefore, in addition to preventing bacteria invading systemically, host defence pathways also apply evolutionary pressure on the microbiota to limit the presence of potentially colitogenic strains.

1.4.2.1 The antimicrobial barrier

The antimicrobial barrier consists of a mucous layer, extending ~100-150µm from the epithelial cell surface. This mucous layer is formed of mucin glycoproteins that are secreted by intestinal goblet cells. The inner portion of the layer, extending approximately 50µm from the surface, traps various classes of antimicrobial proteins secreted by intestinal epithelial cells (IECs), in particular by Paneth cells [172, 181]. These antimicrobial peptides target the bacteria via destruction of the bacterial cell wall or by sequestering vital nutrients such as iron. In this manner a sterile zone is maintained above the epithelial surface with microbes only present in the outer 100µm portion of the mucous [192]. The importance of the mucous layer in preventing undesirable inflammatory responses is clear from the spontaneous inflammation that arises in MUC2^{-/-} mice where the formation of this barrier is compromised and epithelial layer exposed [183].

The renewal of the epithelial layer after damage and the production of some, but not all, antimicrobial peptides is dependent on constitutive “tonic” bacterial stimulation of IECs [185, 193-196]. For example, stimulation through MyD88 or through NLRs, (e.g. Nlrp3 or NOD2), is required for the production of the antimicrobial peptides RegIII γ and α -defensins/cryptidins respectively [194, 197, 198]. NLR-signalling activates the inflammasome, resulting in caspase 1 activation and active IL-1 β and IL-18 production both of which signal through MyD88-dependent mechanisms [199]. The exogenous provision of IL-18 via an osmotic pump or i.p. injection into mice deficient in NLR signalling ameliorates the inherent susceptibility phenotype [188, 191]. Therefore it seems likely that NLR control of host defence can be indirect via IL-18 production. As different antimicrobial peptides target different classes of bacteria, the selective induction of different types of antimicrobial peptides by bacteria in the intestine might be one way by which the composition of the microbiota is modulated.

Complete efficiency of the physical barrier is impossible given the high turnover of the epithelial cell layer and the evolution of many bacteria, such as *Helicobacter* [200] or *Clostridium*-related species [201] that enables them to occupy the niche adjacent to IECs. Furthermore, as the very maintenance of this barrier is dependent on bacterial signals [180, 185, 201] some bacteria or bacterial products must penetrate on rare occasions. Therefore, the intestinal epithelial layer and the underlying lamina propria contain many innate and adaptive cell populations which serve to enhance barrier function and target the occasional invaders.

1.4.2.2 IgA production

A major adaptive host defence mechanism in the intestine is the production of anti-bacterial IgA antibodies by B cells. This is demonstrated by the fact that IgA-deficient mice show systemic IgG responses against components of the microbiota [202]. IgA production can be driven in a T cell dependent manner within the germinal centres of the Peyer's patches or lymph node, or in a T cell-independent manner in isolated lymphoid follicles and in the lamina propria [203]. T cell dependent IgA production is facilitated by T_{FH} cells via the production of IL-6 and IL-21 or by FoxP3⁺ Treg cells which can convert to T_{FH} cells *in vivo* [136, 204]. IgA antibodies are transcytosed across the epithelium and, like antimicrobial proteins, are embedded within the mucous layer preventing bacterial penetration. Precisely how IgA functions is not clear, but presumably IgA binding to bacteria within the mucin glycomatrix physically restricts bacterial translocation and, should the bacteria reach the lamina propria, the opsonisation enhances phagocytosis by resident macrophages and DCs [172, 205]. IgA-producing B cells are absent in the intestines of germ-free mice, but in colonised mice DCs carrying intestinal bacteria are capable of inducing IgA-producing cells [174]. Therefore, intestinal IgA production is also dependent upon the microbiota.

1.4.2.3 Phagocytosis and bacteriolysis

Macrophages and DCs rapidly phagocytose invading bacteria in the lamina propria. It has been suggested that intestinal macrophages rapidly degrade bacteria in lysosomes whereas bacteria is preserved longer in intestinal DCs allowing them to transport antigen to lymph nodes [174]. Interestingly, TLR-ligation of human intestinal macrophages enhances their bacteriocidal activity but does not induce pro-inflammatory cytokine production thus allowing resistance to

infection without initiating an inflammatory cascade [206]. A population of DCs expressing CX3CR1 have some characteristics of macrophages and extend dendritic protrusions between epithelial cells into the lumen [62]. Recent data shows that, after phagocytosing *Salmonella*, CX3CR1⁺ DCs actually transcytose the epithelial layer and exit into the lumen [207] (perhaps explaining the previously observed protrusions). Thus these CX3CR1⁺ DCs prevent invasion by intestinal bacteria. Similarly, CX3CR1-deficient mice, which are unable to form protrusions, are susceptible to *Salmonella* infection reaffirming their role in host defence [62]. In addition, intestinal macrophages can also produce growth factors which act to encourage restitution of the epithelial barrier following damage [208].

1.4.2.4 Cytokine production: IL-17, IL-22 and IL-23

Many populations of innate and adaptive lymphoid cells reside in the intestinal lamina propria and/or the epithelial layer. These include innate lymphocytes (CD8 α ⁺-, $\gamma\delta$ - or $\alpha\beta$ -TCR⁺ cells) [209], natural killer (NK) cells [210], lymphoid tissue-inducer (LTi) cells and innate lymphoid cells (ILCs) [211], as well as adaptive Th17 and Th1 cells [212, 213]. One shared feature of these various cell populations (with the exception of Th1 cells) is the ability to produce IL-22 and IL-17 which are important cytokines for the maintenance of host defence [214]. IL-17 and IL-22 act individually or in synergy to promote antimicrobial protein production [215-218], epithelial barrier integrity [219, 220] and neutrophil and monocyte/macrophage recruitment [216-219]. Their necessary role in host defence is evident from the enhanced susceptibility to *Citrobacter rodentium* infection [215, 221] and the exacerbated chemically-induced intestinal inflammation [214, 222, 223] that is observed in IL-22-deficient and IL-17A-deficient mice. IL-17 and IL-22 production is induced in both innate and adaptive

cells by stimulation with IL-23 and reaffirming the importance for this pathway in host defence IL-23p19^{-/-} mice also show marked susceptibility to *Citrobacter rodentium* infection [118]. In addition to the cell populations indicated above recent data has shown that mice deficient in natural killer T (NKT) cells, which can also produce IL-22 following stimulation [224], have altered microbiota composition. Pre-treatment with the NKT activating ligand α GalCer can protect against DSS-colitis, suggesting that NKT cells contribute to intestinal host defence perhaps by applying evolutionary pressure to the microbiota [225].

The numbers of CD4⁺ T cells present in the lamina propria of germ-free mice are greatly reduced compared to conventionally colonised mice [177]. Furthermore, there is increasing evidence that the differentiation of CD4⁺ T cells is influenced by the composition of the microbiota. Both Th17 and Th1 cells in the lamina propria have been linked to the presence of Segmented Filamentous Bacteria (SFB) which interestingly is able to reside within the “sterile” zone and attach to epithelial cells [201, 226, 227]. It has also been demonstrated that bacterially-derived ATP signals via CD11c^{lo}CD70⁺ DCs to enhance the induction of intestinal Th17 cells [228]. Therefore there is increasing evidence that adaptive CD4⁺ T cell responses involved in intestinal host defence are influenced by the composition of the microbiota.

1.4.3 The maintenance of mutualism: Active tolerance

As shown above the mechanisms of host defence are important in preventing infection and in maintaining a degree of ignorance towards the microbiota. However, many of the same pathways involved in host defence (e.g. IL-23 signalling) are also implicated in driving intestinal inflammation [229]. Therefore, in

order to prevent excessive inflammation, it is vital to limit the responsiveness of the intestinal immune system to these stimuli via mechanisms of active tolerance.

1.4.3.1 TLR hyporesponsiveness

There is evidence that IECs and intestinal macrophages are adapted to limit and manage their responsiveness to bacterial stimuli [230]. IECs express little TLR4, CD14 or TLR2 [231, 232] which reduces their ability to respond to LPS [233, 234]. Similarly, IECs have been shown to internalise TLR4 and TLR2 following stimulation, which subsequently leaves them hyporesponsive to respective TLR ligands [235]. Instead the TLRs and NLRs which are expressed in IECs, as in other cell types, are those localised to intracellular endosomes (e.g. TLR3, TLR7 and TLR9) or the cytoplasm (e.g. NOD2). Furthermore, TLR5, which facilitates recognition of bacterial flagellin and is expressed on the IEC surface, is thought to be localised preferentially to the basolateral membrane and thus basolateral exposure to flagellin elicits a more pro-inflammatory responses [236, 237]. However, this has not been observed in all studies and so remains contentious [231]. Therefore, collectively it is hypothesised that IECs are biased in favour of responding to bacteria which penetrate either intracellularly or across the IEC barrier. Generally invasive ability of this sort is restricted to pathogens whereas non-pathogenic bacteria are limited to the intestinal lumen thus allowing for crude distinction between pathogens and commensals at the level of the innate response.

The responsiveness of IECs to bacteria is further limited by expression of inhibitors of signalling pathways downstream of PRRs [230]. In particular, deletion of SIGIRR, a repressor of IL-1 and TLR signalling, enhances intestinal inflammatory responses [238]. Further downstream of PRR signalling, NF- κ B

activity in IECs is also attenuated. IKK β , which blocks activation of the NF- κ B by preventing nuclear translocation, is protected from degradation in IECs due to the action of commensal bacteria which block its ubiquitination [239]. PPAR- γ , which is expressed at very high levels in IECs, also acts as an inhibitor of NF- κ B mediated inflammation, by binding to and translocating RelA (a subunit of the NF- κ B complex) out of the nucleus [240]. Therefore, it is clear that IECs are uniquely adapted to reduce their responsiveness to bacterial stimuli. In fact members of the microbiota enhance this non-responsiveness. For example, *Helicobacter hepaticus* modulates epithelial cells *in vitro* [241] and *in vivo* [242], possibly via a type VI secretion system, and PPAR- γ expression in IECs is elevated by *Bacteroides thetaiotamicron* [240].

1.4.3.2 Regulatory T (Treg) cells

A requirement for Treg cells in combating the risk of inflammation arising in the intestine is best demonstrated by the T cell transfer model of colitis. In this model the adoptive transfer of naive T cells (i.e. without Treg cells) into SCID recipients results in lymphoproliferation of these cells and the onset of severe colitis [243]. The fact that colitis does not develop after similar cell transfer into germ-free recipients demonstrates that the inflammatory response is driven by the presence of the microbiota [168]. Transfer of CD25⁺ Treg cells either with naive T cells or after disease on-set is sufficient to prevent or ameliorate the inflammatory response [243, 244]. Even under homeostatic conditions in wild-type mice, antigen experienced CD4⁺ T cells with “colitogenic potential” can be found in the MLN suggesting that suppression of commensal reactive CD4⁺ T cells in the GALT is a necessary constitutive process [245]. Treg cells can be separated into two main classes: FoxP3⁺ nTreg cells, which arise “naturally” in the thymus, and FoxP3⁺

iTreg cells, which are “induced” in the periphery [246]. In addition, FoxP3⁻ IL-10-producing CD4⁺ T cells can have significant regulatory function leading to their description as type I regulatory (Tr1) cells [128].

1.4.3.3 Thymically-derived “natural” Treg (nTreg) cells

nTreg cells are generated in the thymus dependent upon TCR stimulation by “self” antigen (likely with affinity lying between that resulting in negative and positive thymocyte selection), CD28 mediated co-stimulation and γ -chain cytokine signalling (predominantly IL-2, but also IL-15 and IL-7) [106]. These signals combine to induce and maintain expression of the “master regulator” of Treg function, FoxP3 [124, 125]. The identification of catastrophic autoimmune phenotypes resulting from FoxP3 mutations in mice and humans indicate the critical importance of nTreg cells in suppressing auto-reactive T cell responses [124, 125, 247-249]. These phenotypes do not just manifest in the intestine, but result in lymphocytic infiltrate of many different organs. Furthermore, studies in FoxP3-deficient mice raised under germ-free conditions have demonstrated that nTreg cells prevent inflammation driven by self-antigen and not only that driven by the microbiota [250]. Therefore, nTreg cells have a broad role in preventing lymphoproliferative auto-inflammation during homeostasis.

1.4.3.4 “Induced” regulatory T (iTreg) cells

Although it is likely that the majority of FoxP3⁺ Treg cells found in lymphoid organs arise in the thymus [251], it is also clear that FoxP3⁺ T cells can be induced from the naive T cell pool (iTreg cells) [105, 252]. This peripheral induction of FoxP3⁺ T cells is enhanced in particular locations and by particular conditions *in vivo*. These include in the GALT [157, 158, 251, 253], following the administration of antigen

[254, 255], in tolerised skin grafts [256] and during inflammation [251]. iTreg cells require a defined TCR-peptide-MHC interaction [257], low CD28 co-stimulation [156] and, critically, TGF- β and CTLA-4 signalling [123, 258] for their development. Notably these latter two necessities contrast significantly with the requirements for the selection of nTreg cells described above.

Once generated it is not possible to easily distinguish iTreg cells and nTreg cells by phenotypic or functional markers. Therefore, it is difficult to assess accurately the relative contribution of iTreg cells to the maintenance of mutualism in the intestine. However, the enhanced conversion of adoptively-transferred naive CD4⁺ T cells into iTreg cells in the GALT of WT or RAG2^{-/-} mice [251, 253] suggests a particular requirement for iTreg cells in the maintenance of tolerance in the intestine. Similarly, iTreg cells are generated in the GALT following the oral administration of antigen, suggesting of a likely mechanism for the establishment of oral tolerance [157, 158, 255]. Lastly, elegant experiments published during the writing of this thesis, have shown that transfer of nTreg cells alone into FoxP3-deficient mice, which by definition are unable to convert existing naive CD4⁺ T cells into iTreg cells, is insufficient to prevent colitis. However, restoring iTreg cell generation in recipient mice by co-transferring FoxP3-sufficient naive CD4⁺ T cells with nTreg cells efficiently prevented colitis indicating the importance of iTreg cell in supplementing the regulatory response in the intestine [259].

1.4.3.5 Treg cell immune suppression in the intestine

The mechanisms by which FoxP3⁺ Treg cells mediate suppression of immune responses are manifold [260] and except in rare occasions it is hard to identify clear functions specific to nTreg cells versus iTreg cells (see below and [252]). The secretion of the cytokines TGF- β , IL-10 and IL-35 have all been shown to be

crucial to the Treg cell-mediated prevention or cure of colitis [261-264]. In addition, cell contact-dependent mechanisms are of clear importance for T cell mediated suppression, with CTLA-4 ligation in particular being paramount. This has been demonstrated by the spontaneous autoimmunity which arises in CTLA-4-deficient mice [265] as well as the effect of antibody-mediated neutralisation of CTLA-4 in alleviating Treg cell-mediated suppression of T cell transfer colitis [266]. Finally, it has recently become apparent that an ability to express certain transcription factors (such as T-bet) or co-stimulatory molecules (such as OX40) has an important role in allowing Treg cells to survive and compete in otherwise polarising environments which might favour effector T cell activity [267].

Secreted cytokines can act on a range of cell types and both TGF- β and IL-10 have been shown to have important CD4⁺ T cell intrinsic functions [262, 268, 269]. However, many actions of Treg cells appear focused on manipulating the function of antigen presenting cells, such as DCs [105]. This concept has particularly arisen following imaging studies which show close contacts between DCs and Treg cells. This interaction is hypothesised to physically exclude effector CD4⁺ T cells *in vitro* [270] and *in vivo* [271] and so prevent T cell activation. Furthermore, and rather contentiously, CTLA-4 has been suggested to function by allowing Treg cells to physically sequester CD80 and CD86 from the surface of DCs via a process referred to as trogocytosis [272].

Interestingly, bacteria in the gastrointestinal tract may enhance regulatory T cell function. It has been suggested that Treg cells isolated from germ-free mice are less suppressive than those isolated from SPF mice suggesting the commensal flora may play a role in modulating the regulatory response [273]. However, this phenomenon has not been reproducible in all published studies [168, 250]. A

possible reason for the conflicting reports may be the presence (or absence) of specific bacteria in the SPF mice of different facilities. Indeed, there is evidence that the presence of specific bacteria, such as *Helicobacter hepaticus* [274], or infection with the human commensal *Bacteroides fragilis* [275, 276], can enhance the suppressive activity of Treg cells. Therefore, the composition of the commensal flora may play a role in modulating the Treg cell response. However, the mechanism for this remains unclear.

1.4.3.6 IL-10

IL-10-deficient mice develop “spontaneous” intestinal inflammation [277] which subsequent studies have shown to be driven by the presence of intestinal bacteria [165, 166] and in particular specific bacteria such as *Helicobacter hepaticus* [278]. Therefore, IL-10 is essential for the prevention of intestinal inflammation. IL-10 was originally described as a Th2 cell cytokine however it is now clear that it can be produced by many cell types, both innate and adaptive, in a context dependent manner [279]. However, the intestinal inflammation that arises in CD4 cell-specific IL-10^{-/-} mice is equivalent to that in complete IL-10^{-/-} mice and therefore CD4⁺ cell derived IL-10 is non-redundant in preventing intestinal inflammation [280]. Myeloid cell-specific (LysM-cre) IL-10^{-/-} do not develop spontaneous inflammation [281]. However, “innate” cell derived IL-10 may assist in the maintenance of tolerance by enhancing FoxP3 expression in CD4⁺ T cells [282]. IL-10 mediates its effect by activating STAT3 downstream of the IL-10R (composed of IL-10R1 and IL-10R2 subunits) and IL-10R-deficient mice and also humans develop an IBD phenotype [283, 284]. Spontaneous intestinal inflammation develops in a monocyte/macrophage specific (LysM-cre) STAT3^{-/-} mice indicating that these cells are the critical targets of IL-10 signalling in the prevention of inflammation [285,

286]. A milder form of enterocolitis is evident in a DC specific (CD11c-cre) STAT3^{-/-} indicating that IL-10 signalling specifically into DCs may have a supplementary role in the maintenance of tolerance [287]. Precisely how IL-10 signalling modulates macrophage and/or DC function is unclear. However, recent data suggests that IL-10 may underlie the hyporesponsiveness of intestinal macrophages/DCs to TLR stimuli [234].

Interestingly, following *H. hepaticus* infection of wild-type mice populations of cells with suppressive capacity could be identified within both the CD25⁺ (regulatory) T cell fraction and, somewhat unexpectedly, within the CD25⁻ T cell fraction [274]. Likewise, CD25⁻CD45Rb^{lo} “antigen experienced” CD4⁺ T cells isolated from the MLN of SPF mice have been shown to have a suppressive capacity [245]. However, unlike for FoxP3⁺ Treg cells which can suppress inflammation by numerous mechanisms, suppression of inflammation by CD25⁻ CD45Rb^{lo} cells was strictly dependent on their ability to produce IL-10 [245, 274]. Mice with a FoxP3⁺ cell specific deletion of IL-10 do develop “spontaneous” inflammation showing that FoxP3⁺ Treg cells are a non-redundant source of IL-10 *in vivo* [288]. However, the phenotype of these mice is milder than that resulting from total CD4 IL-10-deficiency [280] indicating that FoxP3⁻ CD4⁺ T cell derived IL-10 likely has a significant role.

IL-10-producing FoxP3⁺ and FoxP3⁻ CD4⁺ have been identified in the intestinal lamina propria and epithelial layer by antibody staining [263] or using a recently described dual reporter mouse strain [289]. This is consistent with the suggestion that IL-10 from both populations may contribute to tolerance. Similar FoxP3⁻ IL-10-producing CD4⁺ T cells have been described *in vitro* arising from chronic stimulation and/or defined stimuli, such as IL-10 or Vitamin D3 and

Dexamethasone [128, 290]. These cells have been termed T regulatory type 1 (Tr1) cells to take into account their suppressive capacity. However whether they represent a distinct Th lineage or an “exhausted” effector cell remains contentious.

The precise mechanisms which induce IL-10 production from immune cells particularly *in vivo* are poorly understood [279]. However, the role of the IL-12 family cytokine, IL-27, is better characterised than most and interest has grown in its pleiotropic function in recent years [291]. IL-27 is formed of IL-27p28 and EBI-3 subunits and signals through the IL-27R complex formed of the specific IL-27R α subunit and promiscuous GP130 subunit [292-294]. It was first characterised as a more pro-inflammatory cytokine promoting Th1 cell responses by inducing expression of the IL-12R in newly activated CD4⁺ T cells [293-295]. Consistent with a pro-inflammatory function, IL-27R α -deficiency results in the amelioration of T cell transfer- and chemically-induced colitis [296, 297]. However, more recently, a role for IL-27-signalling in promoting IL-10 production has been postulated. Stimulation of newly activated naive CD4⁺ T cells or pre-polarised Th1, Th2 or Treg cells with IL-27 induces IL-10 and transfer of these IL-10 producing cells into a model of CNS-inflammation reduces disease severity [298-303]. Although a possible role for IL-27 in controlling IL-10 production in the intestine remains uncharacterised it is interesting that IL-27R α ^{-/-} mice have elevated proportions of intestinal Th17 cells [304]. This suggests there may be some alteration in intestinal immune-homeostasis in favour of more inflammatory pathways in the absence of IL-27 and indeed in this setting acute chemical-induced inflammation was exacerbated by IL-27R α -deficiency [304].

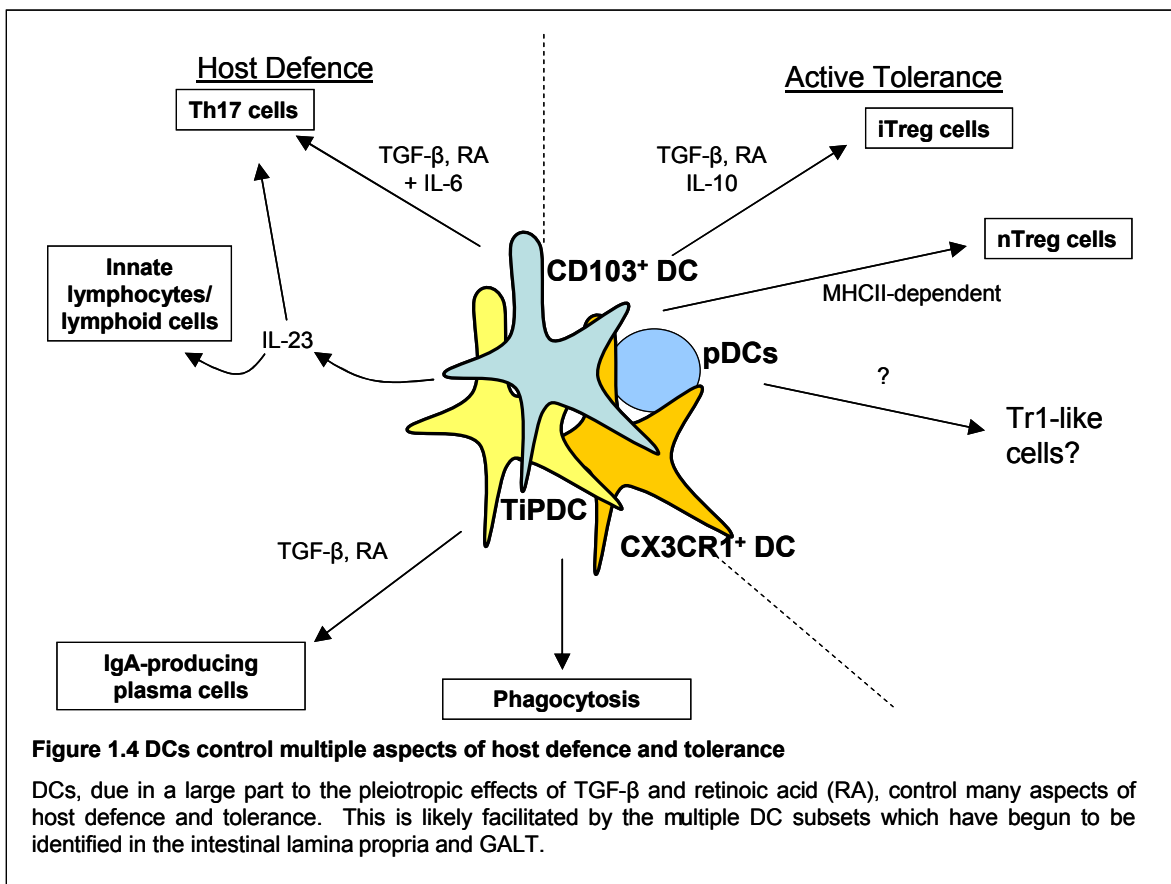
1.4.3.7 Mechanisms of tolerance vary depending on the stimulus

Although the term tolerance is used interchangeably to describe the active inhibition of inflammation in the intestine the precise mechanism likely varies depending on the stimulus. For example, in T cell transfer colitis, which is driven by lymphoproliferation in response to bacterial antigen, expression of the gut-homing integrin $\beta 7$ is dispensable for Treg cell function [305]. However, Treg cell expression of the chemokine receptors CCR4 and CCR7, which facilitate entry into the MLN, are completely essential [306, 307]. Therefore, it seems that in T cell transfer colitis, Treg cells act in the MLN, consistent with a mode of action centred on Treg cells inhibiting naive $CD4^+$ T cell activation by DCs. In contrast, the development of oral tolerance in response to orally administered antigen, which seems to require iTreg cell induction, has a clear requirement for $\beta 7$ expression and thus presumably homing of Treg cells away from the lymph node [308]. In addition in both the development of oral tolerance and in the prevention of T cell transfer colitis IL-10 is dispensable [245, 309]. However, in the maintenance of tolerance against bacteria or in the cure of on-going T cell mediated colitis IL-10 is essentially required [263, 278]. Therefore it is apparent that depending on the stimulus the accompanying mechanism for tolerance appears to differ.

1.5 Intestinal DCs

Dendritic cells in the intestine are key controllers of both host defence and tolerance pathways (**see figure 1.4**). Intestinal DCs promote IgA production and Th17 cell differentiation necessary for host defence [174, 310, 311]. Furthermore, immunohistochemical analysis and the use of an IL-12p40-YFP reporter strain have indicated that DCs in the ileum constitutively produce IL-23 and thus are the major producers of this cytokine during homeostasis [312]. As IL-23 is central to

the IL-22 and IL-17 host defence pathway in both innate and adaptive lymphoid cells IL-23 production by DCs suggests a contribution towards host defence which may be independent of their ability to present antigen. In promoting tolerance, DCs in the small intestinal lamina propria and MLN are capable of enhancing iTreg cell induction and DC migration to the MLN is essential for oral tolerance to be established [157, 158, 313].



How intestinal DCs simultaneously control the development of both effector and regulatory CD4⁺ T cell responses is at present unclear. As described above lymphoid tissue DCs can be separated into functionally distinct subsets and bacterial or host signals can differentially modulate DC maturation resulting in distinct Th cell differentiation. Therefore hypotheses have focused on identifying distinct subsets of intestinal DCs and identifying factors in the intestinal

environment which uniquely “condition” them towards more inflammatory or tolerogenic phenotypes.

1.5.1 Intestinal DC subsets

Several populations of intestinal DCs have been described in the lamina propria and GALT which appear to have distinct functional characteristics [314]. The MLN and Peyer’s patches contain $CD8\alpha^+CD11b^-$ and $CD8\alpha^-CD11b^+$ DCs as described above for the spleen and also include a large population of $CD8\alpha^-CD11b^-$ DCs [315]. $CD8\alpha^-CD11b^+$ DCs isolated from the Peyer’s Patches preferentially promote Th2 cell responses involving elevated IL-4 and IL-10 production from activated $CD4^+$ T cells. In contrast, $CD8\alpha^+CD11b^-$ and double negative DCs preferentially promote Th1 responses [315]. $CD11b^+$ DCs from the Peyer’s patches have also been shown to have enhanced ability to induce IgA production from B cells via IL-6 secretion [316]. In the small intestine and colonic lamina propria DCs appear to be predominantly $CD11b^+$ with the expression of $CD8\alpha$ low and variable between studies [62, 312] [60, 317-320]. However, the phenotype of DCs in colon and small intestine were yet to be fully compared when this study was initiated. A subset of DCs producing TNF- α and expressing inducible-nitric - oxide-synthase (iNOS) has also recently been described in the lamina propria, and to a lesser proportion in the Peyer’s patch and the MLN, indicating a gut-resident population of TiP-DCs present during homeostasis. This population of TiP-DCs was $CD11c^{lo}$ and expressed lower CD86 than conventional DCs, but potently regulated IgA-production from B cells [321]. Finally, the cannulation of rat afferent lymphatic vessels led to the identification of two, presumably migratory, DC populations: $CD4^+/SIRP\alpha^+$ DCs and $CD4^-/SIRP\alpha^-$ negative DCs [322] the latter of

which appear to carry apoptotic epithelial cells and to initiate reduced T cell activation *in vitro* possibly akin to murine CD8 α^+ DCs [64].

Recently, DC subsets have been defined in the MLN and lamina propria based on α_E -(CD103) integrin expression [60, 323]. The proportion of CD103 $^+$ DCs in the MLN is reduced in CCR7 $^{-/-}$ mice suggesting that this is likely to encompass a migratory DC population [323]. However, the presence of CD8 α^+ CD103 $^+$ DCs in the spleen and the fact that not all CD103 $^+$ MLN DCs are absent in the MLN of CCR7 $^{-/-}$ mice suggests that some CD103 $^+$ may also be lymphoid-tissue resident. The evidence that Treg cells fail to protect from T cell mediated colitis in CD103 $^{-/-}$ mice suggested that CD103 expressing DCs might be particularly important for the maintenance of tolerance [60]. Indeed, CD103 $^+$ DCs, isolated from either the MLN or the small intestine, preferentially induce FoxP3 $^+$ iTreg cells from naive T cells, whereas MLN CD103 $^-$ DCs have a comparatively enhanced ability to induce Th1 cells [157, 158]. However, somewhat contradictory to these results, CD103 $^+$ DCs from the small intestine have since been shown to enhance Th17 cell differentiation and intestinal CD11b $^+$ CD11c $^{-/lo}$ cells, termed macrophages, promoted iTreg cell differentiation [311].

Apart from CCR7 expression which is thought to define migratory DCs, intestinal DC subsets have also been defined based on expression of other chemokine receptors: CCR6 and CX3CR1. These chemokine receptors are likely to be important for the localisation of these subsets to specific regions of the Peyer's patches and lamina propria. CX3CR1 $^+$ DCs are localised in the sub-epithelial dome (SED) of the Peyer's Patches and are also dispersed sub-epithelially throughout the lamina propria [62, 324]. From this location, they are primed to take up antigen, for example transcytosed by M-cells, and it is hypothesised that

antigen uptake is enhanced by the extension of dendrites through the epithelial layer in a CX3CR1-dependent manner [62]. CCR6⁺ DCs are also normally localised to the SED of the Peyer's Patches, but are rapidly recruited to the follicle-associated epithelium (FAE) in a CCR6-dependent manner in response to *Salmonella* infection where they internalise bacteria [324]. The enhanced susceptibility of both CX3CR1^{-/-} and CCR6^{-/-} mice to *Salmonella* infection suggest that these DC populations and the ability to respond to these chemokines are important in enabling host defence against infection. Similarly, the location of CD103⁺ DCs has been hypothesised to be of functional importance. CD103 is a ligand for E-Cadherin which is expressed at high levels on epithelial cells. Therefore it has been hypothesised that the interaction of these molecules juxtaposes CD103⁺ DCs to epithelial cells which might be important in the acquisition of antigen or in the conditioning of these DCs prior to maturation [314].

Finally, populations of plasmacytoid DCs have recently been identified in the small intestine, MLN and Peyer's Patches. There is evidence that pDCs in these locations are conditioned to promote Tr1 cell differentiation [325] and also to react to TLR stimuli to promote conventional DC migration to lymph nodes [326].

1.5.2 CD103⁺ DCs, TGF- β , retinoic acid

CD103⁺ DCs have been shown to promote FoxP3⁺ Treg induction via the production and activation of TGF- β and the production of retinoic acid [157, 158]. In fact both TGF- β and retinoic acid are molecules that act in a pleiotropic manner. Therefore the centrality of these molecules in CD103⁺ DC function likely underscores their ability to induce both host defensive and tolerance-mediating intestinal immune responses *in vivo* (see figure 1.4).

TGF- β drives both Treg cell and Th17 cell induction in a concentration dependent manner. High concentrations of TGF- β induce Treg cells [123] and lower concentrations in the presence of IL-6 promote Th17 cell differentiation [116, 117]. Similarly, higher concentrations of retinoic acid enhance Treg cell induction while inhibiting the generation of Th17 cells [156, 327-332], but recent data suggests that low concentrations of retinoic acid may actually enhance Th17 cell differentiation [310]. Also supporting a role for retinoic acid in driving host defence, nutritional deficiency in vitamin A, a precursor of retinoic acid, has long been associated with susceptibility to infectious disease [333].

Both active TGF- β and retinoic acid also play roles in promoting IgA class switching, a second important mediator of host defence [334, 335]. Indeed, IgA-producing B-cells are absent from TGF- β RII-deficient mice [336]. Finally, retinoic acid induces expression of gut-homing receptors on B cells and on both CD4⁺ and CD8⁺ T cells thus directing adaptive immune responses, be they tolerogenic or host defensive, back towards the lamina propria [323, 327, 335, 337-339]. Therefore, the production of TGF- β and retinoic acid by CD103⁺ DCs provides the means to control many aspects of tolerance and host defence in the intestine.

It is intriguing that retinoic acid can be generated from vitamin A or carotenoids that are obtained in the diet. In fact, retinoic acid can in many ways be thought of as an environmental factor present in the intestine. The conversion of retinol to retinoic acid involves two step dehydrogenation and a retinaldehyde intermediate [340]. The first step is catalysed by alcohol dehydrogenases (ADHs) which are ubiquitously expressed. The second step is irreversible and is catalysed by retinaldehyde dehydrogenases (RALDHs), encoded by the ALDH1A family. ALDH1A expression is considerably more tissue and cell-type restricted. CD103⁺

DCs isolated from the MLN, small intestine or Peyer's Patches preferentially express ALDH1A2 (RALDH2) which is likely to underlie their ability to drive retinoic acid dependent processes [157, 338, 341]. Peyer's Patch DCs may also express ALDH1A1 (RALDH1) [338]. Given retinoic acid's central role in controlling a wealth of different immune responses it is perhaps unsurprising that CD103⁺ DCs are not the only intestinal population capable of its production. Indeed, both epithelial cells and MLN stromal cells express ALDH1A enzymes and produce bioactive retinoic acid [342, 343]. The relative contribution of retinoic acid production by different cell types remains, at present, unclear.

Therefore, it is apparent that small intestinal and MLN CD103⁺ DCs form a specialised DC subset characterised by the ability to produce retinoic acid and also drive TGF- β dependent T cell responses. It remains unclear how CD103⁺ DCs are related to other distinct intestinal DC subsets, such as CX3CR1⁺ DCs, or even to intestinal macrophage populations, which may share over-lapping phenotypes and function [344].

1.5.3 TGF- β activation

TGF- β (in particular TGF- β 1) is essential for the maintenance of tolerance as demonstrated by the onset of autoimmunity in TGF- β 1^{-/-} mice [345]. The activity of TGF- β is activity is tightly regulated [346]. Transcription and translation of the TGF- β isoforms (1-3) produces a latent TGF- β complex formed of an active TGF- β subunit bound to the inhibitory latency-associated peptide (LAP). Cleavage by the protease furin ensures that LAP and active TGF- β are secreted as a non-covalently bound latent TGF- β complex. Active TGF- β can be released from this complex by many factors including integrins (α v β 6 and α v β 8), acid, matrix metalloproteases (MMP-1 and MMP-9) and thrombospondin [347]. However, the

fact that both $\alpha\beta6^{-/-}\alpha\beta8^{-/-}$ mice and $\text{TGF-}\beta^{\text{RGE/RGE}}$ (in which $\text{TGF-}\beta$ lacks an integrin binding site, but remains susceptible to other forms of activation) phenocopy $\text{TGF-}\beta1^{-/-}$ mice strongly suggests that activation of $\text{TGF-}\beta$ by integrins is the dominant pathway for immune tolerance *in vivo* [348, 349]. $\alpha\beta8$ expression by DCs in particular is critical for immune homeostasis in the intestine as DC-specific $\beta8^{-/-}$ mice and myeloid-specific $\alpha\beta^{-/-}$ mice develop spontaneous colitis [350, 351]. Therefore, $\beta8$ expression appears to be important for intestinal DCs to maintain tolerance. However, differential expression of $\beta8$ by different DC subsets had not yet been published when this study was initiated.

1.5.4 Intestinal DC conditioning

As noted by Coombes *et al*, it remains unclear whether the unique phenotype of intestinal DCs, most apparent for CD103^+ DCs, is the product of these cells being a distinct DC lineage or a result of conditioning by the intestinal environment [314]. This latter hypothesis stems from evidence that different combinations of microbial and host-derived signals induce different transcriptional and functional responses in DCs. In the intestine, epithelial-derived thymic stromal lymphopoeitin (TSLP) appears to play such a conditioning role, modulating DC phenotype away from IL-12p40 production and towards IL-10 production [352]. This has the result of directing immune responses away from potentially damaging IL-12-driven Th1 responses towards more protective Th2 responses. Hence, $\text{TSLP-R}^{-/-}$ mice as well as mice with impaired TSLP-expression resulting from IEC-specific $\text{IKK-}\beta$ -deletion, show exacerbated Th1/Th17-mediated intestinal inflammation and impaired defence against intestinal helminth infection [353, 354]. Interestingly, limited evidence suggests that TSLP expression might also be reduced in epithelial cells isolated from Crohn's disease patients suggesting a potential

mechanism for the apparent immune-pathology [352]. TSLP-mediated modulation of DCs towards a Th2 cell promoting phenotype is maintained even after exposure to strongly Th1 polarising bacteria [352]. Similar effects limiting pro-inflammatory cytokine expression and DC maturation have been observed for TGF- β and IL-10 and deficiencies in either of these cytokines results in the intestinal inflammation. As mentioned above the requirement for IL-10-signalling in the prevention of colitis is intrinsic to myeloid cells [283, 286]. Therefore, it is conceivable that production of both TGF- β and IL-10 play key DC conditioning roles.

The uptake of apoptotic cells in the intestine is likely to play a substantial role in conditioning DCs and migratory DCs carrying remnants of apoptotic epithelial cells have been detected in rat lymph draining the intestine [64]. Uptake of apoptotic cells results in substantial release of active TGF- β [355] and could therefore contribute to iTreg cell induction during homeostasis. However, apoptosis of epithelial cells during *Citrobacter rodentium* infection results the release of TGF- β and IL-6 which enhances Th17 differentiation and so contributes to disease [356].

There is also limited evidence that conditioning of intestinal DCs can be mediated by the microbiota either directly or indirectly via IECs. In the intestine bacterial ATP has been shown to promote a Th17 cell-inducing phenotype in CD11c^{lo} CD70⁺ intestinal DCs [228]. Similarly, Serum Amyloid A, produced in the intestine in response to SFB, has been shown to promote a similar Th17 cell-inducing DC phenotype at least *in vitro* [201]. Conversely, infection with *Bacteroides fragilis* or treatment with the *Bacteroides*-derived polysaccharide, PSA, enhances FoxP3⁺ Treg cell numbers and their IL-10-production in a TLR2-dependent fashion and thus protects against colitis [102, 275, 276]. It is evident that PSA acts partly through a T cell intrinsic mechanism; however a complementary role acting on

DCs cannot be excluded [102]. Finally, pre-treatment of mice with PRR-ligands such as the NOD-2 agonist, MDP, or the TLR9 agonist, CpG, can be protective against colitis and the latter has been associated with elevated Treg cell responses [285, 357, 358]. However a mechanism for this effect at present remains unclear. Conversely TLR9 agonism from microbiota-derived DNA has been shown to inhibit the induction of iTreg cells in response to oral antigen suggesting it may condition DCs in a more inflammatory manner although again a precise mechanism is lacking [359].

Germ free studies have found limited effects on the mechanics of the DC response *in vivo* with DC migration still intact, little change in DC numbers or the proportion of different DC subsets in the MLN or in the expression of maturation markers [360, 361]. However, there is evidence that DC recruitment to the lamina propria might be induced by microbial colonisation of the intestine [362].

1.6 Inflammatory bowel disease: The causes and consequences

As described throughout this introduction, forward genetic studies in mice have demonstrated that alterations in various aspects of host defence (e.g. PRR deficiency, antimicrobial production) and tolerance (e.g. IL-10-deficiency) can cause a breakdown in mutualism and the onset of intestinal inflammation. However, except in rare occasions (such as FoxP3^{-/-} mice), these mutations do not result in inflammation unless an additional microbial, chemical or immune-activator is present. It should come as no surprise then that genome wide association studies have implicated a substantial array of different genetic polymorphisms with the development of IBD in humans, but that the penetrance (i.e. likelihood of a polymorphism predicting onset of IBD) of each is low [363]. Therefore, IBD is generally thought of as a polygenic disorder, where the onset is likely dependent

on a combination of genetic, microbial, environmental and immune factors [364]. An interesting exception to this rule is indicated by mutations in the IL-10 pathway (specifically IL-10R1 and IL-10R2), which have recently been shown to result in early onset IBD, manifesting before one year of age [284].

Consistent with the diverse array of different “causes” for IBD, the disease itself actually encompasses a range of different clinical manifestations and not all patients show responsive to the same therapies [2]. Currently, IBD is separated clinically into two distinct disorders: Crohn’s disease and ulcerative colitis (UC). Crohn’s disease can manifest in either the colon or small intestine, in sporadic patches, and inflammation often penetrates transmurally. UC is restricted to the colonic mucosa and spreads outwards from an initial flare rather than appearing in patches. In addition, Crohn’s disease is often associated with additional clinical symptoms such as fistulas or granulomas, which again may indicate different inflammatory mechanisms. It is notable that different polymorphisms can specifically associate with one form of the disease but not the other. For example, IL-10 polymorphisms are associated with ulcerative colitis but not Crohn’s disease whereas the reverse is true for NOD2 polymorphisms. This again suggests that different mechanisms underlie the onset of these diseases.

1.6.1 Inflammatory bowel disease: Immune pathways driving inflammation

Despite the array of different factors which can instigate a breakdown in mutualism, they appear to converge on specific immune pathways which subsequently drive inflammation. TNF- α is one common contributor to inflammation in both animal models and many, but not all, patients as indicated by the therapeutic effect of Infliximab, an anti-TNF α antibody [363]. A second cytokine critical for the induction of intestinal inflammation in mouse models is IL-

23, which is formed from IL-12p40 (shared with IL-12) and IL-23p19 subunits. IL-23-deficiency resulting from the genetic deletion of either subunit prevents *Helicobacter hepaticus* induced inflammation in 129RAG^{-/-} mice, anti-IL-10R-treated or IL-10^{-/-} mice, and in T cell transfer colitis [365-368]. Although originally thought of as an inducer of Th17 cells, it seems likely that IL-23 acts more widely promoting both Th17-type responses (including IL-17A, IL-17F and IL-22) and also IFN- γ production from both innate and adaptive cells [212, 368, 369] as well inhibiting the responses of Foxp3⁺ Treg cells [253]. Polymorphisms in both IL-12B (encoding IL-12p40) and the IL-23R have been linked to both Crohn's disease and ulcerative colitis [370, 371] and IL-12p40 biological therapies are undergoing clinical trials [363]. Other cytokines strongly implicated in intestinal inflammation in mouse models are IL-6, which may act in synergy with IL-23 to promote Th17-type responses, and the inflammasome effector cytokines, IL-1 β and IL-18, acting downstream of NLRs such as NLRP3. Accordingly, polymorphisms in genes encoding signalling molecules downstream of the IL-6R (e.g. STAT3 and Jak2) are associated with Crohn's disease and UC and polymorphisms in Nlrp3 and an IL-18 receptor accessory protein (IL-18RAP) are associated with Crohn's disease [363]. However, STAT3 is involved in the downstream signalling pathways of numerous receptors including, notably, the IL-10R and polymorphisms in the NLRs, Nlrp3 and NOD2, appear to cause reduced IL-1 β production. Thus, these pathways require further study to determine precisely how they act in contributing to inflammation.

1.6.2 Inflammatory bowel disease: The contribution of the microbiota

As indicated throughout this introduction the microbiota is an essential component of many mouse models of IBD [165-168, 278]. In addition, specific bacterial

species strongly influence the development and severity of intestinal inflammation but altering CD4⁺ T cell function [274, 275, 278]. In fact, emerging data suggests that this influence of the microbiota may not be limited to the intestine but affects a diverse range of inflammatory conditions such as arthritis [372], CNS-inflammation [373], asthma [374, 375] and diabetes [376]. The microbiota exerts this effect by modulating the immune system in ways described throughout and also by providing antigen to drive adaptive effector T cell responses. In addition, fermentation of dietary factors by the microbiota can release beneficial short chain fatty acids (SCFAs), such as butyrate, which act via G-protein coupled receptors (GPRs), such as GPR43, to maintain homeostasis although the precise downstream effects are unclear [377]. Thus the diet and the microbiota can combine to modulate the immune response [378].

Concurrent with the emergence of the microbiota as a key component in animal models, improvements in sequencing technology have allowed detailed analysis of the composition of the human microbiota (by 16s rRNA sequencing) and their genetic components (by metagenomic analysis), referred to as the microbiome. These studies have indicated that there is remarkable variability in microbiota composition between individuals of different families, but that it is fairly stable within the same individual over time [163]. Metagenomic analysis has indicated that, although diverse combinations of different bacterial species can form a stable microbiota, what is important is the collective ability of these species to carry out a core set of functions (e.g. carbohydrate metabolism) required to survive in the intestinal niche [379]. The microbiota of monozygotic twins is not substantially more similar than in dizygotic twins suggesting that “non-genetic” factors are primary determinants of the composition of the microbiota and it is likely that

maternal “inheritance” is the strongest of these [380]. However, the composition of the diet can alter the intestinal environment to the advantage or disadvantage of different bacterial species. Indeed, recent data suggests that alteration of the composition of the microbiota by the diet can have therapeutic benefit in animal models of colitis [381, 382].

Alterations in the composition of the human microbiota, perhaps caused by antibiotic treatment or more hygienic living conditions, have been hypothesised to underlie the increased prevalence of human metabolic and inflammatory disease. However, it remains unclear whether these changes are really a cause or consequence of disease progression. Most notably, obese individuals and genetically-susceptible obese mice contain a microbiota reduced in *Bacteroides* species and increased in *Firmicutes* which facilitate more efficient calorific intake from the diet. Accordingly, transplantation of this altered microbiota into wild-type mice resulted in significant weight gain suggesting that the metabolic contribution of the microbiota might contribute to the development of obesity [383]. In IBD patients, there is an overall reduction in microbial diversity in the intestine with a proportional reduction in *Firmicutes* and *Bacteroides* phyla and an increase in *Enterobacteriaceae* [384]. Furthermore an absence of the bacteria *Faecalibacterium prausnitzii* correlated with a greater chance of Crohn’s disease patients relapsing [385]. Finally, a direct comparison of twins with IBD showed that the composition of the microbiota is altered specifically in Ileal Crohn’s disease suggesting a specific impact for the microbiota in the development of inflammation at this site [386]. There is therefore substantial therapeutic interest of using probiotics or prebiotics to reverse microbial alterations and, optimistically, restore intestinal homeostasis. Indeed, perhaps as a crude fore-runner to pro-

biotic and pre-biotic therapy, whole “microbiota transplant” has been successfully implemented as a means of restoring the defence against *Clostridium difficile* infection in otherwise susceptible patients[387].

1.7 Summary

In conclusion, intestinal inflammation is caused by a breakdown in the ability of the immune system and the microbiota to maintain mutualism. Both host defence and tolerance mechanisms are important in conserving mutualism and significantly intestinal DCs are uniquely specialised to regulate these apparently opposite processes. In particular, CD103⁺ DCs promote both host defensive IgA-production and Th17 cell differentiation [310] as well as more tolerogenic iTreg cell differentiation [157, 158]. Underlying CD103⁺ DC function is their ability to produce and activate TGF- β and to produce retinoic acid, both of which have pleiotropic effects in immunity. However, how CD103⁺ DCs are regulated and how they relate to other intestinal DC populations, such as CX3CR1⁺ DCs remains unclear.

Regulatory T cell (Treg) responses are critical for the maintenance of tolerance. There is clear emerging evidence that effector CD4⁺ T cell responses in the intestine can be modulated by the alterations in the microbiota. However, how the microbiota modulates intestinal DC subsets and Treg cell responses also remains unclear.

1.7.1 The aims and approach of this thesis

The aims of this thesis were three-fold.

- 1) Characterise CD103⁺ and CX3CR1⁺ DCs in the GALT, small intestine and colonic lamina propria. In particular focusing on the CD103⁺ DCs with high ALDH enzyme activity thought to correlate with retinoic acid production.
- 2) Develop an *in vitro* system for CD103⁺ DC differentiation to indicate growth factors required for ontogeny and “conditioning” of this DC subset
- 3) Determine the relationship between the microbiota, intestinal DC subsets and regulatory CD4⁺ T cell activity. In particular focusing on the ability of DCs to induce FoxP3⁺ Treg cells and Treg cell production of IL-10, two critical components of tolerance.

These aims were approached in the following ways.

- 1) Multiparameter flow cytometry was employed to identify and phenotype populations of CD103⁺ DCs and CX3CR1⁺ cells in the GALT and intestinal lamina propria under homeostatic and inflammatory conditions. This study is described in chapter 3.
- 2) The generation of CD103⁺ DCs was assessed in bone marrow-derived DC cultures (BMDCs) using combinations of the growth factors FLT3L and GM-CSF which are known to induce DC development. This study is described in chapter 4.
- 3) The proportions and character of intestinal DC subsets and regulatory T cells were assessed following alteration of the microbiota either by broad spectrum antibiotics or by infection with *Helicobacter hepaticus*. In particular, the effect on the production of IL-10 by Treg and Tr1-like cells in the GALT was studied in detail. This study is described in chapter 5.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Mice

Balb/c, $\alpha_E(\text{CD103})^{-/-}$, Ly9.2, DO11.10 SCID, C57Bl/6 (B6), CX3CR1^{GFP/GFP} 'knock-in', B6.MyD88^{-/-}, FoxP3^{huCD2/huCD2}, IL-10^{GFP/GFP} 'tiger' and MyD88^{-/-}.IL-10^{GFP/GFP} mice were maintained as in-bred lines under SPF conditions in an 'in house' accredited facility (University of Oxford). CX3CR1^{GFP/+} mice were generated by breeding CX3CR1^{GFP/GFP} with B6 wild-type mice. All experiments were performed in accordance with the United Kingdom Animals Scientific Procedures Act (1986).

All *in vivo* protocols conducted in this thesis including animal handling, mouse breeding, genotyping, intraperitoneal (i.p.) and intravenous (i.v.) injections, oral gavage, antibiotic treatment, schedule one termination and the dissection of tissues was conducted by the author occasionally in collaboration with other lab members.

2.1.2 Antibodies

The following panels of directly conjugated fluorescent anti-mouse monoclonal antibodies were used for flow cytometric analysis of different cell populations. All antibodies were used at concentration 1-2 $\mu\text{g/ml}$ unless otherwise indicated. The optimal combinations of antibodies used for identifying and isolating myeloid cells in different settings are indicated in section 2.2.3.

CD11c-PerCPCy5.5, CD11c-APC and CD11c-PECy7 (HL/3), I-A/I-E-APC and I-A/I-E-efluor450 (MHCII) (used at 0.2µg/ml) (M3/114.15.2), CD40-APC (3/23), CD80-APC (16-10A1), CD86-APC (GL1), ICOS-PE (7E-17G9), CD103-APC (2E7), ICOS-L-PE (HK5.3), PD-1-PE (2GF1A12), PD-L1-PE (M1H5), PDL-2-PE (TY25), CD8α-APC (53-6.7), CD4-APC and CD4-PEC7 (GK15/RM4-5), TCR-β-PerCPCy5.5 (H57-597), DO.11.10TCR-APC (KJ1.26), FoxP3-efluor450 (FJK-16S) (eBioscience, UK)

CD11b-APC (M1/70), CCR7-APC (4B12), E-Cadherin-FITC (36), CTLA-4-PE (UC10-4F10-11) (BDbiosciences, UK)

Ly6C-PECy7 (HK1.4) (used at 0.5µg/ml) (Biolgend, UK)

IL-27R-PE (used at 10µg/ml) (R&D systems, Abingdon, UK)

Rat IgG1 (R3-34) (BD Pharminogen), Rat IgG1 (eBRG1) (eBioscience), Rat IgG2a (R35-95) (BD Pharminogen), Rat IgG2b (A95-1) (BD Pharminogen) conjugated to the relevant fluorochromes as appropriate.

For *in vivo* studies the following antibodies were purified by affinity chromatography (anti IgG column) from hybridoma supernatant; Anti-PDCA-1 (120G8, kindly provided by G. Trinchieri [388]), anti-IL-10R (1B1.2). Antibodies were endotoxin tested (Lonza, Verviers, Belgium, <0.1 EU/mg) prior to administration.

Hybridoma cell lines were cultured in the cultivation chamber of a CELLline flask (Beckton Dickinson, Oxfordshire, UK, product code CL-1000) in 20ml hybridoma media. Hybridoma media consisted of RPMI 1640 supplemented with 10% low Ig FCS (Gibco, Invitrogen, Paisley, UK, product code 16250-078, lot number 830345) and 20ml of integra supplement per 500ml media. Integra supplements were

made up separately by Chris Jenkins consisting of 60g/litre peptone (from meat, Sigma-Aldrich, Type I, product code P7750), 75g/l D-(+)-Glucose (Sigma-Aldrich, Altringham, UK), 40mM L-glutamine, 333U/ml Penicillin + 333µg/ml Streptomycin (Sigma-Aldrich, Altringham, UK), 30% (vol: vol) nonessential amino acids (Sigma-Aldrich, Altringham, UK, product code M175), 300µl/l 1% β-Mercaptoethanol (Sigma-Aldrich, Altringham, UK). The nutrient supply chamber of the CELLline flask was filled with 500ml RPMI 1640 supplemented with 20ml integra supplements. To maintain optimal antibody production cells were split twice per week; 12ml of cells were removed and replaced with 12ml fresh hybridoma media. Media in the nutrient supply chamber was aspirated and 500ml fresh RPMI 1640 supplemented with 20ml integra supplements was also provided. To harvest supernatant containing antibody, the 12ml cells were centrifuged for 8 minutes at 1300rpm and the supernatant transferred to a fresh 50ml falcon tube. The supernatant was then centrifuged at 3000rpm for 10 minutes without a brake and stored at -20°C prior to antibody purification.

2.1.3 Bioactive molecules

Lipopolysaccharide (LPS) from *E. coli* (Axxora, California, USA. Product code, ALX-581-007), CpG-ODN (Invivogen, California, San Diego, product code ODN 1585) and polyI:C (Sigma Aldrich, Altringham, UK, Product code P1530), all-trans retinoic acid (Sigma-Aldrich, Altringham, UK, product code R2625).

2.1.4 General reagents

The following reagents were used in various reaction buffers and cell culture protocols described below. They are listed here for convenience.

RPMI 1640 (Sigma-Aldrich, Altringham, UK), phosphate buffered saline (PBS, PAA laboratories, Yeovil, UK) or from PBS tablets (Dulbecco A, Oxoid, Hampshire, UK, product code BR0014G), bovine serum albumin (Fraction V, PAA laboratories, Yeovil, UK, product code K45001), penicillin-streptomycin (Sigma-Aldrich, Altringham, UK, product code, 031M0787), L-glutamine (Sigma-Aldrich, Altringham, UK, product code G7513), fetal calf serum (FCS, Sigma-Aldrich, Altringham, UK, product code F9665, majority from lot 030M3399), HEPES (Sigma-Aldrich, Altringham, UK), β -mercaptoethanol (>98%, Sigma-Aldrich, Altringham, UK)

Polypropylene falcon tubes (Beckton Dickinson, Oxfordshire, UK, 15ml product code 352096, 50ml product code 352070)

ACK lysis buffer (used for red blood cell lysis section 2.2.1): 0.15M NH_4Cl , 10mM KHCO_3 , 0.1mM Na_2EDTA dissolved in H_2O and pH adjusted to 7.2-7.4 using HCl. Filter sterilized prior to use.

Digest media (used in section 2.2.1): RPMI 1640 / 10%FCS / 7.5mM HEPES

LPL EDTA media (used in section 2.2.2): RPMI 1640 / 5%FCS / 100U/ml penicillin / 100 $\mu\text{g}/\text{ml}$ streptomycin / 5mMEDTA

LPL wash media (used in section 2.2.2): RPMI 1640 / 5%FCS / 100U/ml penicillin / 100 $\mu\text{g}/\text{ml}$ streptomycin

LPL collagenase media (used in section 2.2.2): RPMI 1640 / 5%FCS / 100U/ml penicillin / 100 $\mu\text{g}/\text{ml}$ streptomycin / 0.5mg/ml collagenase Type II from *Clostridium histolyticum* (Sigma-Aldrich, Altringham, UK product code C6885) / 40 $\mu\text{g}/\text{ml}$ DNase I from bovine pancreas (Roche, Burgess Hill, UK, product code 11284932001)

MACS buffer (used in section 2.2.3): PBS / 0.1%BSA / 2mM EDTA

Complete cell culture media (used in section 2.3.4): RPMI 1640 / 10%FCS / 100U/ml penicillin + 100µg/ml streptomycin / 2mM L-glutamine / 0.05mM β-mercaptoethanol.

Tryptone soya broth (used in section 2.4.3 for *Helicobacter hepaticus* culture): 30g/litre Tryptone soya broth dissolved in 1000ml H₂O and autoclaved. 10% FCS and 2ml campylobacter selection antibiotics (Oxoid, product code SR0069E).

2.2 Cell isolation

2.2.1 Isolation of leukocytes from lymphoid tissues

Spleen, mesenteric lymph nodes, inguinal lymph nodes and/or Peyer's patches were dissected and adipose tissue or surrounding intestinal tissue was carefully removed. Organs were cut into small pieces using a scalpel blade and transferred to 15ml polypropylene falcon tubes (Beckton Dickinson, Oxfordshire, UK) with 4ml Digest media (see section 2.1.4) supplemented with 0.75mg/ml collagenase type VIII (Sigma-Aldrich, Altrincham, UK, product code C2139). Tissue was incubated with agitation at 200rpm at 37°C for 35 minutes. At the end of the digest 200µl of 0.5M EDTA was added directly to the cell suspension to quench collagenase activity. The digested material was then passed through a 70µm cell strainer (Beckton Dickinson, Oxfordshire, UK) and remaining tissue physically disaggregated using a 2ml syringe (Beckton Dickinson, Oxfordshire, UK). The cell strainer was then rinsed with 5ml PBS / 0.1%BSA and the resulting cell suspension transferred to a fresh 15ml falcon tube. Cells were centrifuged at 1350rpm for 5 minutes. The cell pellet was resuspended in 5ml PBS/0.1%BSA prior to cell counting.

For splenic cell suspensions, red blood cells were lysed by incubation in 0.5ml ACK lysis buffer (see section 2.1.4) for 3 minutes at room temperature.

2.2.2 Isolation of leukocytes from colonic/caecal/small intestinal LP

The protocol used to extract leukocytes from the colonic lamina propria was substantially optimised over the course of this thesis. Comparisons have only been drawn between data acquired using the same protocol. Listed here is the optimised protocol used for the majority of experiments (adapted from [228]). Colon, caecum and small intestine were dissected and adipose tissue was carefully removed. Peyer's patches were also excised from the small intestine using a scalpel blade and forceps and analysed separately where appropriate (using the protocol described in section 2.2.1).

Contents were forced from the colon using forceps prior to cutting longitudinally and washing three times by submersion and shaking in PBS/0.1%BSA to further remove contents. Clean colon tissue was blotted on a paper towel to remove excess mucous. The small intestine was cut longitudinally with the contents inside. The contents were removed by repeated submersion and shaking in PBS/0.1%BSA and the clean tissue blotted on paper towel. The caecum was cut longitudinally along the inner seam and then cut vertically at either end. The cut caecum was then shaken vigorously in a 1.5ml eppendorf containing 1ml PBS/0.1%BSA to remove contents. The caecal tissue was then submerged in clean PBS/0.1%BSA and shaken to further remove contents prior to blotting on paper towel.

All tissues were then cut laterally into approximately 2cm long pieces and transferred to cold LPL wash media (see section 2.1.4) To remove epithelial cells,

tissue was placed in 50ml falcon tubes, and shaken at 200rpm for 30 minutes in pre-warmed LPL EDTA media (see section 2.1.4). Supernatant was then aspirated and the process repeated. To remove residual EDTA, tissue was incubated in 10ml LPL wash media at room temperature for 10 minutes and the supernatant subsequently discarded. The tissue was then digested by incubation in 20ml LPL collagenase media (see section 2.1.4) in 50ml falcons. In rare occasions, surface markers are cleaved by different collagenase enzymes. This was the case for PDL-1 and so analysis was often repeated exchanging Type II collagenase for Type VIII collagenase (Sigma-Aldrich, Altrincham, UK, product code C-2139) (adapted from [389]) however this reduced cell viability. The addition of 1mg/ml dispase (Gibco) to LPL collagenase media increased the cell yield and viability, but showed evidence of cleaving CD4 from the cell surface and therefore was used for DC cell analysis (as suggested by [228]) but not analysis of CD4⁺ T cell populations. Following tissue digestion the supernatant was passed through a 70µm cell strainer into a 50ml falcon and the volume increased to 30ml with cold LPL EDTA media to quench the collagenase activity. Supernatant was centrifuged at 1400rpm for 5 minutes to pellet cells and supernatant was discarded by aspiration.

Cells were resuspended in 3ml 30% percoll (P30) and layered onto gradient consisting of a 3ml 70% percoll (P70) layer and a 4ml 40% percoll (P40) layer in a 15 ml falcon. Gradients were centrifuged at 1800rpm for 20 minutes at 10°C without a brake. Red blood cells pellet at the bottom of the P75 later, lamina propria leukocytes (LPL) collects at the P75-P40 interface, epithelial cells at the P40-P30 interface and cellular debris rises to the top of the P30 layer. Therefore LPLs were collected by removal of the P30 layer and then aspiration of the cells at

the P75-P40 interface using a plastic Pasteur pipette. Cells were washed two times in cold PBS/0.1%BSA to remove residual percoll prior to flow cytometry (see section 2.3.3) or further cell purification (see section 2.2.3).

2.2.3 DC isolation by FACS

Cell suspensions were prepared from lymphoid tissue (MLN or spleen) or intestinal tissue as described in section 2.2.1 and 2.2.2. Alternatively, CD103⁺ and CD103⁻ DCs were purified from BMDC cultures described in section 2.3.1. For purification of MLN DCs, cell suspensions were pre-enriched for CD11c⁺ cells by positive selection using magnetic beads. MLN cells were suspended at 1×10^8 cells/ml in MACS buffer (see section 2.1.4.) with 5µg/ml anti-mouse CD16/32 'Fc block' (eBioscience, Hatfield, UK clone 93) for 10 minutes on ice to reduce non-specific antibody binding. 30µl/ml anti-CD11c microbeads (Miltenyi Biotech, Surrey, UK) were added directly to the cell suspension and cells were incubated for a further 15 minutes at 4°C. Cells were washed by the addition of 20ml MACS buffer and centrifugation at 1350rpm for 5 minutes at 4°C. Cells were resuspended at 1×10^8 /ml and filtered through a 70µm cell strainer. The cell suspension was then passed through a magnetised LS column (Miltenyi-Biotech, Surrey, UK) by gravity filtration enabling CD11c⁺ cells bound to beads to collect within the column. The column was washed 3 times by passing 3ml MACs buffer through it by gravity filtration. The column was then transferred to a new 15ml falcon tube and CD11c⁺ cells removed by flushing 2ml MACS buffer through the tube using a plunger. This process was repeated twice to flush any remaining CD11c⁺ cells from the column. Such pre-enrichment was not practical for intestinal DC isolation due to the variable level of CD11c expression and the low LPL cell number or from BMDCs which were already mostly CD11c⁺. Cell suspensions were then incubated with

Aldefluor and/or fluorescent antibodies against surface markers (as described in sections 2.3.3 and 2.3.4). The following panels of antibodies and dyes were found to be optimal.

MLN DCs: Aldefluor, CD103-PE, CD11c-PerCPCy5.5, MHCII-APC, DAPI

Intestinal DCs: CX3CR1^{GFP}, CD103-PE, CD45PerCPCy5.5, CD11c-PECy7, MHCII-APC, viability dye efluor780.

BMDCs: CD103-PE, CD11c-PerCPCy5.5, MHCII-APC, DAPI

Cells were resuspended in PBS / 0.1%BSA / 5mM EDTA for FACS isolation to reduce cell aggregation. FACS isolation of specific subsets was achieved to high purity using a FACSaria (BD) or a MoFlo sorter (DAKO cytometry). Sorting was carried out by Helen Ferry (FACSaria, BRC flow cytometry unit, Oxford) or Nigel Rust (MoFlo, Sir William Dunn School of Pathology, Oxford).

2.2.4 CD4⁺ T cell Isolation [157]

Splenic or lymph node cell suspensions were generated as described in section 2.2.1. CD4⁺ T cells were isolated by negative selection using a Dynal Mouse CD4 negative isolation kit (Invitrogen, Paisley, UK, product code 114.15D) using a modified version of manufacturer's instructions. Cells were re-suspended at 2×10^8 cells/ml in PBS / 0.1%BSA and $20\mu\text{l}/10^7$ cells of Dynal antibody mix were added. Cells were then incubated for 20 minutes on ice. Cells were then washed by addition of 20ml PBS / 0.1%BSA and centrifugation at 1350rpm for 5 minutes. Cells were resuspended at 1×10^8 cells/ml in PBS / 0.1%BSA and transferred to a 14ml round bottomed falcon tube (Beckton Dickinson, Oxfordshire, UK, product code 352059). An equal number of Dynal M450 sheep anti-rat IgG magnetic beads to cells were suspended at 1×10^8 /ml in PBS / 0.1%BSA. These were

mixed with cells and incubated with rotation at 4°C for 20 minutes. Non-CD4⁺ cells that were bound to the magnetic beads were removed by placing the falcon tube against a magnet (Invitrogen, Paisley, UK) for 30 seconds. The negative fraction containing CD4⁺ cells was collected by pouring into a fresh 50ml falcon tube. The beads were then resuspended in 7ml PBS / 0.1%BSA and returned to the magnet for 30 seconds. Again the negative fraction cells were collected by pouring into the 50ml falcon. The pooled negative fractions were centrifuged at 1350rpm for 5 minutes and the cells resuspended in 5ml PBS / 0.1%BSA prior to cell counting.

In some experiments naïve CD4⁺ CD45Rb^{hi} T cells were isolated by a further round of positive selection. Briefly, CD4⁺ cells were resuspended at 1 x 10⁸ cells/ml in MACS buffer (see section 2.1.4) with 10µg/ml FITC-conjugated anti-mouse CD45Rb antibody (clone C363.16A, eBioscience, Hatfield, UK) for 20minutes on ice. Cells were then washed by addition of 20ml MACS buffer and centrifugation at 1350rpm for 5 minutes. Cells were resuspended at 1 x 10⁸ cells/ml in MACS buffer with 6µl/10⁷ cells of anti-FITC magnetic beads (Miltenyi Biotech, Surrey, UK) for 20 minutes at 4°C. CD45Rb⁺ cells bound to beads were isolated by passing the cells through a magnetised LS column (Miltenyi Biotech, Surrey, UK) as previously described for CD11c⁺ cell isolation (see section 2.2.3).

2.3 *In vitro* experiments

2.3.1 Bone marrow-derived DC cultures

Bone marrow cells were harvested from the tibia and fibular by flushing with cold PBS using a 5ml syringe and 26G needles under sterile conditions. Cells were filtered into a 50ml falcon and centrifuged at 1300rpm for 5 minutes. Red blood cells were lysed by resuspension in 1ml ACK lysis buffer (see section 2.1.4) and

incubation for 3 minutes at room temperature. Cells were washed twice by addition of 40ml cold RPMI 1640 / 2% FCS and centrifugation at 1300rpm for 5 minutes. Cells were then resuspended in 10ml of complete cell culture media (see section 2.1.4) and counted. Cells were then cultured in the following conditions in 6-well tissue culture plates (Costar, product code 3516).

GM-CSF mediated differentiation [390]

1×10^6 cells/ml were cultured in 3ml of complete cell culture media supplemented with 20ng/ml GM-CSF (Peprotech, London, UK). On day 3, cultures were provided with a further 3ml of complete cell culture media containing 20ng/ml GM-CSF. On day 6, 3 ml of culture media was replaced with fresh complete cell culture media containing 20ng/ml GM-CSF. BMDCs (>80% of the culture expressed CD11c) were analysed between day 7 and day 10.

FLT3L mediated differentiation (adapted from [391])

2×10^6 cells/ml were cultured in 3ml complete cell culture media supplemented with 200ng/ml FLT3L (Peprotech, London, UK) in a 6 well plate. On day 4, cultures were provided with a further 3ml of complete cell culture media containing 200ng/ml FLT3L. BMDCs (>80% of culture expressed CD11c) were analysed between days 8 and 10).

FLT3L/GM-CSF mediated differentiation

BMDCs were generated as for FLT3L mediated differentiation. However, on day 6 cell cultures were supplemented with 20ng/ml GM-CSF (with minimal disturbance of differentiating cells). BMDCs were analysed on day 8.

2.3.2 CD4⁺ T cell and DC co-cultures

Naïve CD4⁺ T cells were isolated either by negative selection of CD4⁺ T cells from lymphoid tissue of DO11.10SCID mice or by magnetic selection of CD4⁺CD45Rb⁺ cells as described in section 2.2.4. CD4⁺ T cells were incubated with 2mM CFSE (Invitrogen, Paisley, UK) at room temperature for 10 minutes so as to control for changes in cell proliferation. CFSE labelling was quenched by addition of 2ml FCS to cell suspensions and cells were washed 3 times in PBS / 2%FCS prior to cell culture. CD103⁺ and CD103⁻ DCs were isolated from MLNs or BMDC-cultures by FACS as described in section 2.2.3. In an antigen specific assay (used for strains on a Balb/c background) 2 x 10⁵ DO11.10 SCID CD4⁺ T cells were incubated with 3 x 10⁴ DCs in complete cell culture media (see section 2.1.4) with 200ng/ml OVA₃₂₃₋₃₃₉ peptide. In a polyclonal assay (used for strains on a B6 background) 2 x 10⁵ CD4⁺ T cells were co-cultured with 3 x 10⁴ DCs with 0.5µg/ml soluble anti-CD3e antibody (154-2C11, ebioscience, Hatfield, UK). Where appropriate cultures were further supplemented with TGF-β at either 2ng/ml or 10ng/ml (R&D Systems, Abingdon, UK). All cultures were conducted in round bottomed 96 well plates (Costar, product code 3799), at 37°C, 5%CO₂. CD4⁺ T cells were analysed for surface markers and FoxP3 expression by flow cytometry (as described in section 2.3.3) after 3 days of culture.

2.3.3 Flow cytometry

1 x 10⁵ – 10⁶ cells were incubated in 50µl PBS / 0.1%BSA with 5µg/ml anti-mouse CD16/32 (93, ebioscience) 'Fc block' in a round-bottomed 96 well plate for 10 minutes at 4°C to reduce non-specific binding. In order to assess cell viability cells were incubated for 30 minutes at 4°C with 1/1000 dilution of either Live-dead aqua (Invitrogen, Paisley, UK) or fixable viability dye efluor 780 (ebioscience, Hatfield,

UK) in 50µl PBS. Cells were washed twice with 150µl PBS / 0.1%BSA to remove residual dye prior to cell surface staining. Alternatively, if cells remained unfixed, cell viability was assessed by incubation with 7-AAD or DAPI (Sigma-Aldrich, Altringham, UK) for 10 minutes at room temperature prior to data acquisition.

For cell surface staining cells were incubated with relevant monoclonal anti mouse antibodies (clones and concentrations listed in section 2.1.2) in 50µl PBS/0.1%BSA for 30 minutes at 4°C. In the majority of experiments, especially those utilising GFP reporter signalling, cells washed with PBS/0.1%BSA and data acquisition began no more than two hours following completion of the staining.

For intracellular Ki67 and/or FoxP3 staining, cells stained with surface markers were washed and incubated in 100µl fixation/permeabilisation buffer (ebioscience, Hatfield, UK) at 4°C overnight. Cells were then washed three times in 200µl of permeabilisation buffer (ebioscience, Hatfield, UK) diluted 1:10 from concentrate with PBS / 2%FCS / 0.01%NaN₃. Cells were then incubated with 50µl diluted permeabilisation buffer supplemented with 1µg/ml anti-mouse CD16/32 'Fc block' for 10 minutes on ice. A further 50µl of diluted permeabilisation buffer containing relevant antibodies was then added and cells incubated on ice for 60 minutes. Cells were washed twice with diluted permeabilisation buffer prior to data acquisition.

Data were acquired on a DAKO Cyan (Dako, Ely, UK) or BD LSRII (BD, Oxford, UK) flow cytometer. If fewer than four markers were assessed, data was acquired on a BD FACScalibur (BD, Oxford, UK) flow cytometer. Compensation was carried out initially using splenocytes stained with the relevant antibodies and subsequently using compensation beads (anti-rat/hamster or anti-mouse Ig κ, BDbiosciences, Oxford, UK, product codes 552845 and 552843). CX3CR1^{GFP} and

IL-10^{GFP} were compensated using small intestinal or colonic lamina propria cell populations. Compensation of live-dead aqua or fixable viability dye efluor780 was conducted using splenocytes. Compensation was carried out by the author and checked relative to fluorescence minus one (FMO) controls where cells were stained with the full panel of antibodies minus the fluorochrome of interest.

2.3.4 Aldefluor assay (adapted from [341])

All reagents purchased as an Aldefluor assay kit (Stem Cell Technologies, Grenoble, France, product code 01700). Aldefluor substrate was acid-activated according to manufacturer's instructions. Prior to antibody staining, cell suspensions were incubated at 1×10^5 - 10^6 cells per ml in 100 μ l assay buffer supplemented with 150nM Aldefluor substrate for 30 minutes at 37°C in a 96 well round bottomed plate. This concentration of Aldefluor substrate was optimal based upon a titration using MLN cells. As a negative control, cells were incubated with 150nM Aldefluor substrate in the presence of 100 μ M diethylaminobenzaldehyde (Deab) (Sigma-Aldrich, Altringham, UK) an ALDH inhibitor. Cells were then washed two times in cold Aldefluor assay buffer before being stained with monoclonal antibodies against cell surface markers as described in section 2.3.3. Importantly all subsequent antibody staining was carried out in Aldefluor assay buffer rather than PBS / 0.1%BSA to prevent Aldefluor product being exported out of the cell.

2.3.5 Determining relative gene expression by quantitative-PCR

RNA was extracted from purified cell populations or whole tissue using RNeasy minikit (Qiagen, Crawley, UK) according to manufacturer's instructions. For cell analysis, cells were lysed in RLT buffer supplemented with 1% β -mercaptoethanol

and stored at -80°C until RNA could be extracted. For tissue analysis, small sections (~0.3cm) of proximal, mid and distal colon were pooled into a 2ml cryotube (Sarsedt, Leciester, UK) and snap frozen in liquid nitrogen. Samples were stored at -80°C until RNA could be extracted. Once thawed, tissue was placed in homogeniser tubes containing ceramic beads (MPBio, Illkirch, France) with 600µl RLT buffer containing 1% β-mercaptoethanol. Tissue was then homogenised by 30 seconds agitation using an HP fastprep24 homogeniser (MPBio).

RNA concentration and purity was measured using a nanodrop. A maximum of 1µg of RNA (quantity was normalised across samples) was converted to cDNA by incubating 12µl RNA with 0.5µl oligo dT primers (Invitrogen, Paisley, UK) and 1µl dNTP (10mM, Bioline, London, UK) at 65°C for 5 minutes and subsequently on ice for 1 minute. This was followed by addition of 0.5µl SuperScript III reverse transcriptase (Invitrogen, Paisley, UK), 4µl first strand buffer (Invitrogen, Paisley, UK), 1µl DTT (Invitrogen, Paisley, UK) and 1µl RNase OUT (Invitrogen Paisley) and incubation for 60 minutes at 70°C.

Q-PCR reactions were carried out using Quantitect Primer Assays (Qiagen) and SYBR green in Sensimix (Bioline, London, UK, product code 650) except for the following where primers were designed in house (sequences denoted 5'-3'). These were conducted in qPCR master mix (Eurogentec, Seraing, Belgium, product code RT-QP2X-03). Primers were used at a concentration of 0.3µM and the probe at 10µM:

IL-27p28: (FWD) ATCTCGATTGCCAGGAGTGA

(REV) GTGGTAGCGAGGAAGCAGAGT

(PROBE) FAM-TCCAGGCTCCCAACTCCACCA-TAM

EBI-3: (FWD) GCCATGCTTCTCGGGTATCC

(REV) GAGCCTGTAAGTGGCAATGA

(PROBE) FAM-TTCCCAATGTTTCCCTGACTTTCCA-TAM

IL-10: (FWD) GCCACATGCTCCTAGAGCTG

(REV) CAGCTGGTCCTTTGTTTCAA

(PROBE) FAM-CGGACTGCCTTCAGCCAGGTG

IL-12p35: (FWD) TACTAGAGAGACTTCTTCCACAACAAGAG

(REV) TCTGGTACATCTTCAAGTCCTCATAGA

(PROBE) FAM-AGACGTCTTTGATGATGACCCTGTGCCT-TAM

IL-23p19: (FWD) CCATCTGGATGATATAGTGATA

(REV) GTCCTAGTAGGGAGGTGTCAAGTTG

(PROBE) FAM-CCAGTTCTGCTTGCAAAGGATCCGC-TAM

HPRT: (FWD) GACCGGTCCCGTCATGC

(REV) TCATAACCTGGTTCATCATCG

(PROBE) FAM-ACCCGCAGTCCCAGCGTCGGTG-TAM

All samples were run in triplicate in 96 well microplates (Bioline) using either a Chromo4 thermocycler (MJ Research) or a C100 thermocycler with a CFX 96 RT system (Biorad). Gene expression was normalised to HPRT, averaged and the relative gene expression was quantified using the $2^{-\Delta C(t)}$ method [392].

2.3.6 Quantification of cytokine production by intestinal myeloid cells

Myeloid cells were isolated as described in section 2.2.3 and cultured overnight at a density of 6×10^5 cells/ml in a minimum of 50 μ l complete cell culture media (see section 2.1.4). Cells were then centrifuged at 1400rpm for 5 minutes and supernatant harvested into a fresh 96 well round bottomed plate. Supernatant was stored at -20°C prior to analysis. The concentration of cytokine in supernatant was determined using a fluorescent bead based assay (eBioscience, UK). Briefly, “simplex kits” were purchased containing anti-cytokine capture beads and biotinylated anti cytokine antibodies used for quantification for the following cytokines: KC, MCP-1, IL-6, MIP-1 α , IL-10, IL-27, IL-23, IL-18, IL-1 β , IFN- γ , TNF- α , GM-CSF. 25 μ l supernatant was incubated with 25 μ l of capture beads (1/80 dilution in assay buffer) and 50 μ l of biotinylated anti-cytokine antibody (1/80 dilution in assay buffer) in a 96 well multiscreen filter plate (Millipore, Watford, UK, product code MBVN1256) and incubated for 2hrs at room temperature with agitation in a 96 well round. Beads were washed by addition of 150 μ l of assay buffer followed by removal by vacuum filtration. This step was repeated. Beads were then incubated with 50 μ l of streptavidin-PE (1/375 dilution) in assay buffer for 1hr at room temperature with agitation. Fluorescence was measured using a FACScalibur flow cytometer (BD, Oxford, UK) and concentration of cytokine determined relative to a standard curve.

2.4 *In vivo* experiments

2.4.1 Modelling the response to orally administered antigen (adapted from [157, 393])

In order to assess CD4⁺ T cell proliferation and iTreg cell induction in response to oral antigen, 5 x10⁶ naïve OVA₃₂₃₋₃₃₉-specific CD4⁺ T cells (Ly9.1⁺), harvested from the peripheral lymph-nodes of DO11.10 SCID mice, were transferred into recipient mice by i.v. injection in 200µl PBS using 0.5ml Insulin syringe (Terumo Medical, Surrey, UK). Starting on the day after transfer, mice which were fed ovalbumin protein (Grade VI, Sigma-Aldrich, Altringham, UK, Product code A2512), from which the OVA₃₂₃₋₃₃₉ peptide is derived, either in the drinking water (20mg/ml for 5 days) or alternatively by oral gavage (50mg/ml on two consecutive days). After 5 days cells were isolated from the MLN, Small intestine lamina propria and Peyer's patches cells as described in section 2.2.1 and 2.2.2. Transferred cells were identified by flow cytometry as Ly9.1⁺ DO11.10TCR (KJ1.26)⁺ cells and the proportion of Ki67⁺ and FoxP3⁺ determined.

2.4.2 Antibiotic treatment

Mice on a C57Bl/6 background were treated with ampicillin sodium salt (1mg/ml, Sigma-Aldrich), neomycin sulfate (1mg/ml, Fisher Scientific), metronidazole (1mg/ml, Sigma-Aldrich), gentamicin (1mg/ml, Sigma-Aldrich) and vancomycin hydrochloride (0.5mg/ml, ACROS) similar to that which has been previously described [185]. Mice received soft chow diet for the first two weeks to limit initial weight loss and dehydration however by three weeks mice were fully recovered.

Alternatively, mice on a Balb/c background were less resistant to weight loss and dehydration. Therefore these mice received a modified protocol of the same antibiotics provided in 200µl by daily gavage for two weeks [394].

2.4.3 *Helicobacter hepaticus* cell culture and infection

H. hepaticus NCI-Frederick isolate 1A (Strain 514449, American Type Culture Collection, Manassas, VA) was grown in minimal oxygen high nitrogen in pressurized glass containers. Initially, bacteria were grown at 37°C on blood agar (Oxoid) supplemented with laked horse blood (Oxoid) and trimethoprim (5µg/ml), vancomycin (10µg/ml) and polymyxin B (25 IU/ml) antibiotics (Oxoid). After 2 days, bacteria were transferred to liquid culture consisting of tryptone soya broth media supplemented with the same antibiotics and 10% FCS. A starting bacterial density of 0.05-0.1 OD₆₀₀/ml was used in 200ml vented plastic Erlenmeyer flasks (Corning). Liquid culture was restored to low oxygen growth conditions in pressurized containers and incubated at 37°C on a shaking incubator. OD₆₀₀ measurements were taken every 24 hours and the culture was diluted to 0.1OD to maintain optimal growth conditions. Viability was assessed every 24 hours using a fluorescent live/dead assay (Invitrogen, Paisley, UK) and an Axioscop2 fluorescent microscope (Zeiss). Only cultures that with high viability and motility were used for infection.

To infect mice, bacteria were harvested by ultracentrifugation at 7000rpm for 15 minutes. The pellet was washed in PBS and then resuspended at 5 OD₆₀₀/ml corresponding to approximately 5×10^8 bacteria/ml in PBS. Mice were fed $\sim 1 \times 10^8$ bacteria in 200µl by oral gavage on three consecutive days starting on day 0 to ensure infection. Fresh bacteria were harvested fresh for each infection.

To investigate whether live *H. hepaticus* was required to mediate immune effects *H. hepaticus* was heat-killed by treatment at 95°C for 15 minutes and the equivalent of 2×10^8 bacteria were administered by gavage.

2.4.4 *Helicobacter hepaticus* induced colitis

In order to initiate intestinal inflammation, mice were infected with *H. hepaticus* as described above and treated with 1mg anti-IL-10R (1B1.2) blocking antibody by i.p. injection using a 0.5ml insulin syringe (Terumo medical, Surrey, UK) on day 0, 7, 14 and 21 as appropriate [365].

2.4.5 Plasmacytoid DC depletion

In order to deplete pDCs *in vivo* mice were treated three times per week separated by 48 or 72 hours with 150µg/ml antiPDCA-1 antibody (120G8) [388] in PBS by i.p. injection in keeping with preliminary data and literature protocols [395]. FoxP3^{huCD2}IL-10^{GFP/+} mice were depleted of pDCs for a two week period prior to the analysis of FoxP3 and IL-10 expression by intestinal CD4⁺ T cells. Non-depleted control mice received i.p. injections of PBS alone. In experiments where pDC depletion was followed by *H. hepaticus* infection, mice were injected twice prior to infection and maintained on 48 hour thrice weekly injections afterwards for a further 14 days.

2.4.6 Statistical analysis

Significance of differences within data sets was determined using a Mann Whitney test or Student's *t*-test on Graphpad Prism software.

Chapter 3

The characterisation of intestinal CD103⁺ and CX3CR1⁺ myeloid cell subsets

3.1 Introduction

Through their ability to sense the local environment and interact with other cells of the immune system DCs are thought to play a key role in regulating the intestinal immune response. In the intestine, they appear to balance the conflicting demands of host defence with the maintenance of tolerance towards the commensal flora and dietary antigen. Although the precise mechanisms and signals which dominate this process remain largely uncharacterised, there are two non-mutually exclusive hypotheses for which supportive evidence is emerging [19, 314]. Firstly, the presence of distinct DC subsets in the intestine that are “naturally” more or less disposed to promote tolerance or inflammation. In this way, subsets promoting tolerance dominate under homeostasis, but more inflammatory subsets expand in response to pathogens or other insults and this is sufficient to allow inflammation to be instigated. Secondly, it is hypothesised that DCs are modulated by the intestinal environment, in a way that “conditions” them towards more pro- or anti- inflammatory phenotypes depending on the precise signals present. Such signals would enhance or reverse the natural phenotype of a DC and tune the resultant immune response more closely to changes in the intestinal environment.

A distinct population of DCs has been identified in the intestinal lamina propria and MLN by expression of the α_E -integrin, CD103 [60, 157, 158, 323]. Subsequently a similar population of CD103⁺ DCs has been identified in the human small intestine

[396]. This CD103⁺ DC population released fewer pro-inflammatory cytokines in response to TLR-stimuli and was better able to promote the regulatory T cell responses associated with oral tolerance suggesting it may represent a “conditioned” DC subset [157, 158]. The more “tolerogenic” nature of CD103⁺ DCs was due to their specialised ability to produce active TGF- β and retinoic acid, leading to the hypothesis that these were features imprinted by conditioning in the intestinal environment [314]. Such conditioning may be mediated by dietary factors, bacterial factors or by the host epithelium [352, 397, 398]. Intriguingly, all trans retinoic acid is produced by metabolism of retinol (vitamin A) which itself can be derived from the diet [343]. The metabolism of vitamin A to retinoic acid occurs via sequential oxidation catalysed by alcohol dehydrogenases (ADH), which are ubiquitously expressed in all cells, and aldehyde dehydrogenases (ALDH), whose expression is considerably more cell type-restricted [340]. CD103⁺ DCs were shown to have high expression of the ALDH enzyme, ALDH1A2 (encoding the protein RALDH2), relative to CD103⁻ DCs in the MLN [157, 158] and also compared to DCs in other locations such as the peripheral lymph nodes [338] and spleen (our own unpublished data). It is therefore likely that high ALDH1A2 expression underlies the ability of intestinal CD103⁺ DCs to utilise retinoic acid.

Retinoic acid has other functions in the intestine which extend beyond that of enhancing FoxP3⁺ Treg cell induction [156, 327-332]. Retinoic acid also induces the expression of gut-homing receptors, such as CCR9 and $\alpha_4\beta_7$, which thus direct CD4⁺ and CD8⁺ T cells and B cells back to the mucosa [323, 327, 335, 337-339]. Also, retinoic acid likely has a role in promoting non-regulatory CD4⁺ T cell responses in more inflammatory settings, for example after stimulation with flagellin [310] or vaccination [399, 400] and retinoic acid also acts to promote IgA

class switching indicating a role in maintaining host defence against the commensal bacteria [310, 335].

In addition to CD103, a second marker, CX3CR1, has been suggested to define intestinal DCs in the Peyer's patches and the intestinal lamina propria. CX3CR1 expression endows DCs with the ability to extend dendrites between epithelial cells which may be a mechanism to sample antigen from the lumen [62]. At the beginning of this study the relationship between CX3CR1⁺ and CD103⁺ DCs had not been determined. We therefore sought to better characterise intestinal CD103⁺ DCs by their ability to produce retinoic acid and to clarify the relationship between CX3CR1⁺ and CD103⁺ DC populations within the lamina propria.

3.2 Results

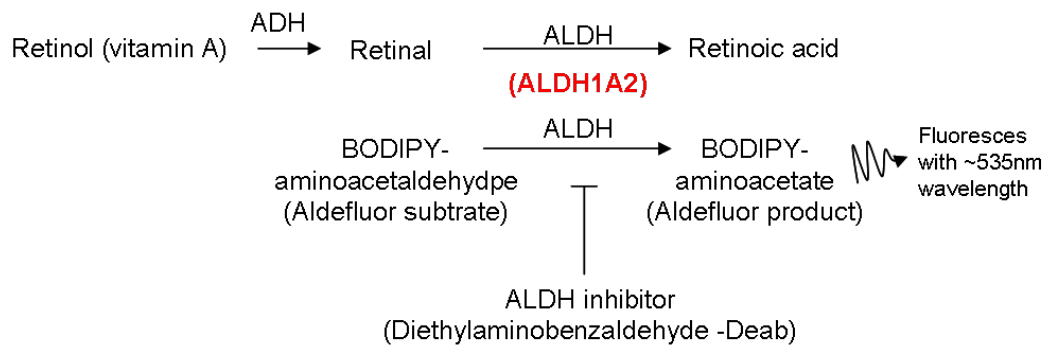
3.2.1 The use of Aldefluor to detect ALDH⁺ DCs in the MLN

Although the identification of CD103 as a marker for a conditioned DC subset was a significant advance, the presence of CD103⁺ DCs in non-intestinal lymphoid tissue, such as the spleen and inguinal lymph nodes, showed that this was not an intestinal-specific DC population [59, 401]. Therefore we sought to further define the CD103⁺ DC population to indicate whether the functional properties of TGF- β activation and retinoic acid production were specific to intestinal CD103⁺ DCs. A fluorescent assay, known as Aldefluor, had been developed to identify stem cells in the bone marrow by ALDH enzyme activity (Stem Cell Technologies). In brief, in the presence of active ALDH enzymes a mildly fluorescent aldehyde substrate (BODIPY-aminoacetaldehyde) is converted to a highly fluorescent acetate product (BODIPY-aminoacetate), modelling the rate limiting step in retinoic acid production

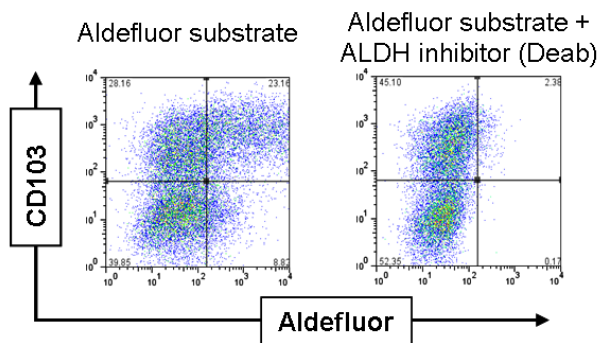
(**Figure 3.1A**). We utilised this assay to attempt to identify CD103⁺ ALDH⁺ DCs in the intestine.

Highly fluorescent, Aldefluor⁺ DCs could be detected in the MLN all of which were CD103⁺. A small proportion of Aldefluor⁺ CD103⁻ DCs could be detected but at a substantially lower level of fluorescence than shown for CD103⁺ DCs (**Figure 3.1B, 3.2A**). The Aldefluor⁺ signal was clearly specific for ALDH activity as the inhibitor of ALDH activity diethylaminobenzaldehyde (Deab) was sufficient to block the development of fluorescence. Interestingly, only ~50% of CD103⁺ DCs in the MLN were positive for Aldefluor indicating that the CD103⁺ DC population in the MLN is heterogeneous in its ALDH activity. We further validated the assay by isolating the Aldefluor⁺CD103⁺ DCs, Aldefluor⁻CD103⁺ DCs and CD103⁻ DCs by FACS and analysing ALDH1A2 expression. ALDH1A2 was only expressed at high levels within the Aldefluor⁺ CD103⁺ DCs indicating the Aldefluor expression correlated with expression of the ALDH enzyme catalysing retinoic acid production (**Figure 3.1C**).

A) Principle of the Aldefluor assay



B) Application of the Aldefluor assay



C) Validation of the Aldefluor assay

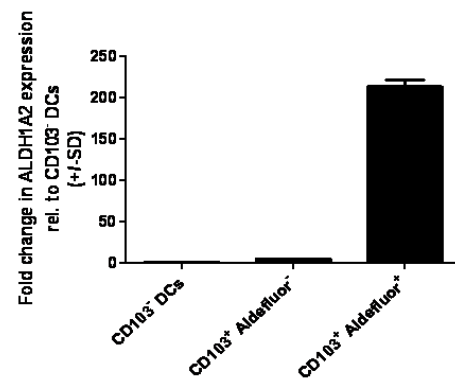


Figure 3.1 Application and validation of the Aldefluor assay

- A) The principle of the Aldefluor assay in modelling the ALDH-catalysed step of retinoic acid production. This step is likely to be driven by RALDH2 activity encoded by the gene ALDH1A2 which is highly expressed in CD103⁺ DCs.
- B) Representative flow cytometry plot showing Aldefluor activity in MLN DCs. Analysis shows CD103 expression against Aldefluor activity. Plots were pre-gated on live (7-AAD⁻) CD11c^{hi} cells.
- C) CD11c⁺ cells were enriched from MLN cell suspensions by MACS. Cells were incubated with Aldefluor substrate and subsequently with fluorescent-conjugated antibodies against CD11c and CD103. CD103⁻, CD103⁺Aldefluor⁻ and CD103⁺Aldefluor⁺ DCs were purified by FACS, RNA was extracted and the expression of ALDH1A2 determined by Q-PCR. Data represents the mean +/- standard deviation of 3 replicates from one experiment. A second experiment gave similar results.

3.2.2 Characterisation of Aldefluor⁺ DCs in the MLN

The surface phenotype of Aldefluor⁺ DCs (**Figure 3.2B**) showed that they could not be distinguished based on the expression of CD8 α or CD11b, markers commonly associated with DC subsets in lymphoid tissue. Instead Aldefluor⁺ DCs expressed intermediate levels of CD8 α and consisted of both CD11b⁺ and CD11b⁻ populations. In addition they did not express the macrophage marker F4/80. Importantly, analysis of MHCII and co-stimulatory molecule expression indicated that Aldefluor⁺ DCs were MHCII^{hi}, CD86^{hi}, CD80⁺ and so represent a highly mature DC population. Previous preliminary data suggested that CD103⁺ DCs had higher expression of the non-conventional MHC-I molecule CD1d, however this was not supported by flow cytometry staining and was not restricted to the Aldefluor⁺ DCs. Interestingly, the vast majority of Aldefluor⁺ DCs were CCR7⁺, a chemokine receptor associated with the migration of DCs from peripheral tissues into the T cell zones of lymph nodes and the subsequent induction of oral tolerance [313, 318]. Therefore, CD103⁺Aldefluor⁺ DCs represent a mature, likely migratory DC population. However, the presence of CCR7⁺Aldefluor⁻ DCs, most of which were still CD103⁺, indicates that ALDH activity is not an absolute property of CCR7⁺ DCs. Finally, the expression of the molecule PDL-2 has very recently been linked to the development of oral tolerance [402]. Given that CD103⁺ DCs promote regulatory T cell induction after administration of dietary antigen [157] we analysed the expression of PDL-2 on Aldefluor⁺ DCs. The vast majority of Aldefluor⁺ DCs expressed PDL-2 and the remaining PDL-2⁺ DCs were all CD103⁺ firmly linking PDL-2 expression to the CD103⁺ DC subset.

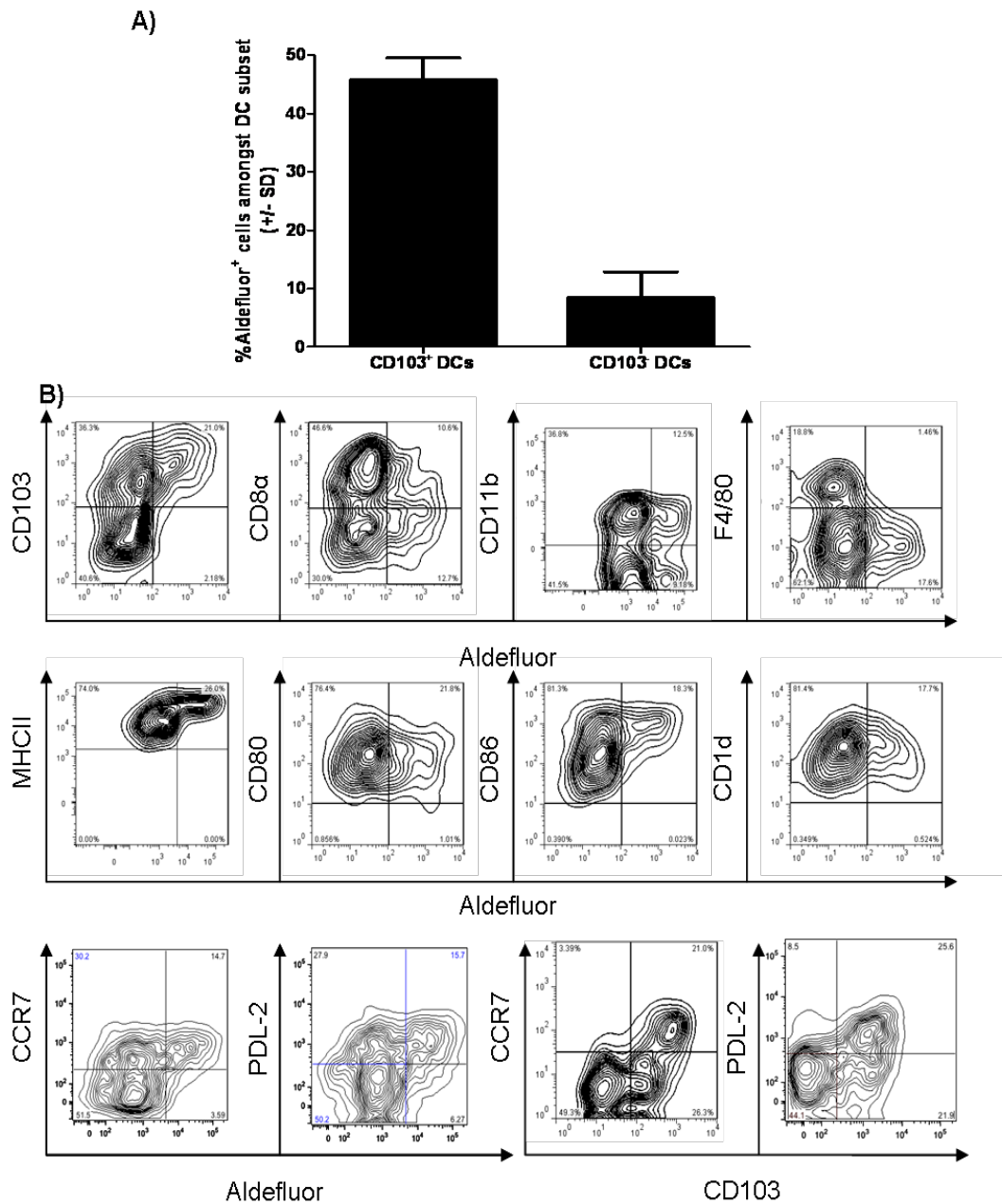


Figure 3.2 The characterisation of Aldefluor⁺ DCs in the MLN

- A) Proportion of Aldefluor⁺ DCs amongst CD103⁺ and CD103⁻ DC subsets was determined by flow cytometry. Data represents the mean \pm SEM of n=8 biological replicates from two independent experiments.
- B) Single cell suspensions were prepared from the MLN by collagenase digest. Relative expression of surface markers on Aldefluor⁺ DCs in the MLN was determined by incubation with Aldefluor substrate followed by relevant antibodies and flow cytometric analysis. All plots are pre-gated on live (7-AAD⁻) CD11c^{hi} cells. Analysis of CD86, MHCII, CCR7, PDL-2, CD11b and CD8α is representative of n=6 replicates from two independent experiments. Analysis of F4/80, CD80, CD1d is representative of n=3 from one experiment.

3.2.3 Expression of TGF- β , β 8-integrin and ability to promote FoxP3⁺ Treg cell induction

TGF- β has been linked to the ability of CD103⁺ DCs to promote iTreg cell differentiation. Therefore, we analysed the expression of TGF- β 2, which had previously been shown to be elevated within CD103⁺ DCs in the MLN, and the TGF- β activating integrin, β 8, which is required for the maintenance of tolerance [350]. TGF- β 2 expression was increased in Aldefluor⁺ DCs, relative to CD103⁻ DCs but not compared to CD103⁺Aldefluor⁻ DCs suggesting that this is a feature of CD103⁺ DCs in general. By contrast, expression of β 8 was enriched amongst Aldefluor⁺ DCs than either CD103⁺Aldefluor⁻ DCs or CD103⁻ DCs suggesting that the ability to activate TGF- β correlates with the ALDH activity (**Figure 3.3A**).

Given the evidence that CD103⁺ DCs promote iTreg cell differentiation, we investigated the relative ability of CD103⁺Aldefluor⁺ DCs, CD103⁺Aldefluor⁻ and CD103⁻ DCs to convert naive DO11.10 CD4⁺ T cells into FoxP3⁺ cells *in vitro*. CD103⁺Aldefluor⁺ DCs were able to induce higher proportions of FoxP3⁺ CD4⁺ T cells than either population of Aldefluor⁻ DCs, particularly in the presence of exogenous TGF- β (**Figure 3.3B**). This indicates that the ability to preferentially induce Treg cells is enriched amongst ALDH⁺ DCs in the MLN, in spite of the fact that these DCs are highly mature.

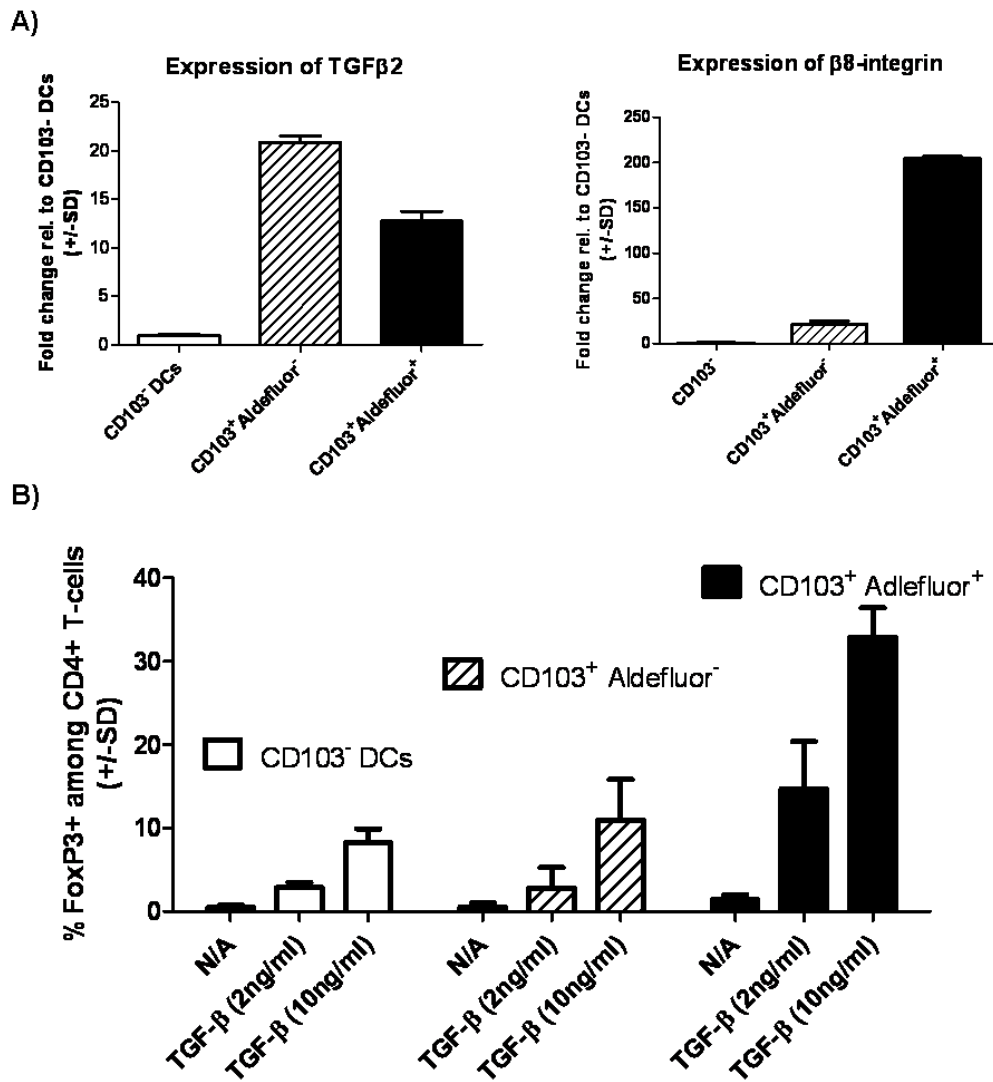


Figure 3.3 Aldefluor⁺ DCs are primed to activate TGF- β and preferentially induce FoxP3⁺ CD4⁺ T-cells *in vitro*

CD103⁻, CD103⁺Aldefluor⁻ and CD103⁺Aldefluor⁺ DCs were isolated from the MLN by CD11c⁺ cell enrichment (MACS) and purified by FACS.

- A) RNA was extracted from purified DCs and the expression of relevant genes determined by Q-PCR. Data represent the mean \pm SD of three replicates from one experiment. A second experiment gave similar results.
- B) The proportion of live CD4⁺TCR- β ⁺ cells expressing FoxP3 was determined by flow cytometry following three days of culture with the specified DC population isolated from the MLN by FACS. Data represent the mean \pm SD from two independent experiments. A third experiment gave an similar pattern of FoxP3 expression (i.e. substantially increased in CD103⁺ Aldefluor⁺ cultures) from a higher base-line.

3.2.4 Aldefluor⁺ DCs are still present and FoxP3⁺ Treg cells are still induced by orally-administered antigen in CD103-deficient mice

The failure of transferred CD25⁺ CD4⁺ Treg cells to protect from T cell transfer colitis in CD103-deficient mice was one of the main indications that CD103 has a functional role on non-T cells, culminating in the identification of specialised CD103⁺ DCs in the MLN [60]. However, the functional role of CD103 remains unclear. The ligand for CD103, E-Cadherin, is expressed at high levels on intestinal epithelial cells and therefore it was hypothesised that the interaction between CD103 and E-Cadherin juxtaposed DCs and epithelial cells and enabled DC conditioning to occur. As Aldefluor activity was associated with highly mature, migratory DCs which preferentially induced iTreg cells, it seemed that it was a good indicator of intestinally-conditioned DCs. Therefore, we investigated whether the proportion of Aldefluor⁺ DCs or the level of Aldefluor fluorescence was reduced in CD103-deficient mice. The proportion of DCs in the MLN and small intestine that were Aldefluor⁺ and the MFI of Aldefluor⁺ DCs was not altered in CD103-deficient mice indicating that the induction of ALDH activity in DCs is not dependent on expression of CD103 (**Figure 3.4**).

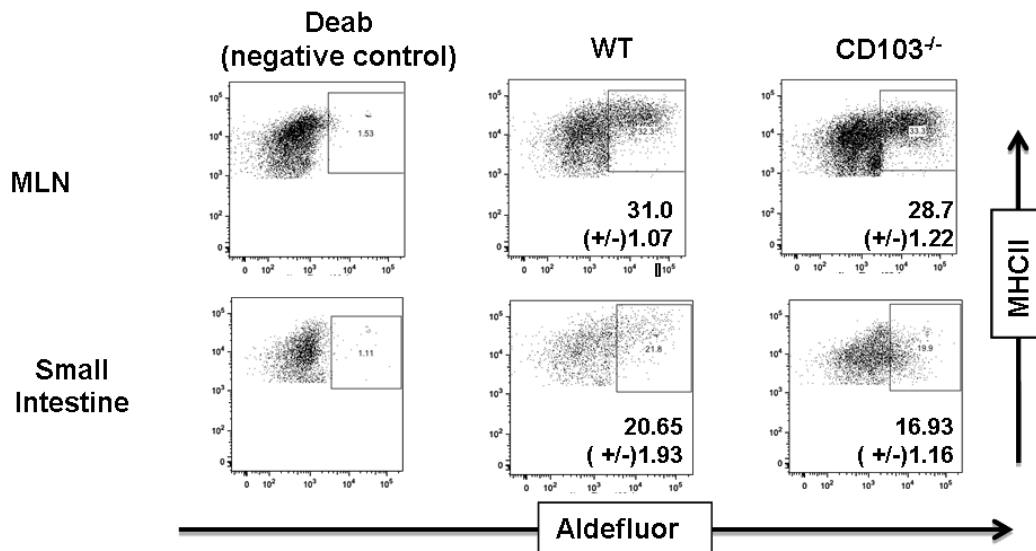


Figure 3.4 Aldefluor⁺ DCs develop normally in CD103^{-/-} animals

Single cell suspensions from MLN or small intestinal LPLs were generated by collagenase digest. Cells were incubated with Aldefluor substrate followed by relevant antibodies. All plots are pre-gated on live, CD11c^{hi}MHCII⁺ cells. Gating set to a negative control containing ALDH inhibitor Deab.

Data representative of n=4 biological replicates from one experiment. Plots are annotated with the mean +/-SEM.

In order to further investigate intestinal DC activity in the absence of CD103, we investigated the immune response to orally administered antigen which is dependent on migratory DCs [13, 313]. OVA₃₂₃₋₃₃₉-specific naive CD4⁺ T cells from DO11.10SCID mice were adoptively transferred into CD103-deficient or wild-type recipients and ovalbumin, which contains the cognate epitope, was administered in the drinking water. The antigen-specific response of the transferred cells in each setting could be observed by pre-gating on CD4⁺ KJ1.26⁺ T cells (**Figure 3.5A**). The administration of ovalbumin induced clear proliferation of the cells in the MLN, Peyer's patches and small intestinal lamina propria, as determined by the presence of Ki67⁺ cells amongst the transferred cell population, that were absent in mice receiving control drinking water. In addition, there was the emergence of a small but significant proportion of FoxP3⁺ KJ1.26⁺ T cells (**Figure 3.5A**). Importantly, there was no defect in the generation of either Ki67⁺

or FoxP3⁺ CD4⁺ T cells in CD103-deficient mice indicating that the ability of DCs to induce proliferation and iTreg cell differentiation in response to orally-administered antigen is independent of CD103 expression (**Figure 3.5B**).

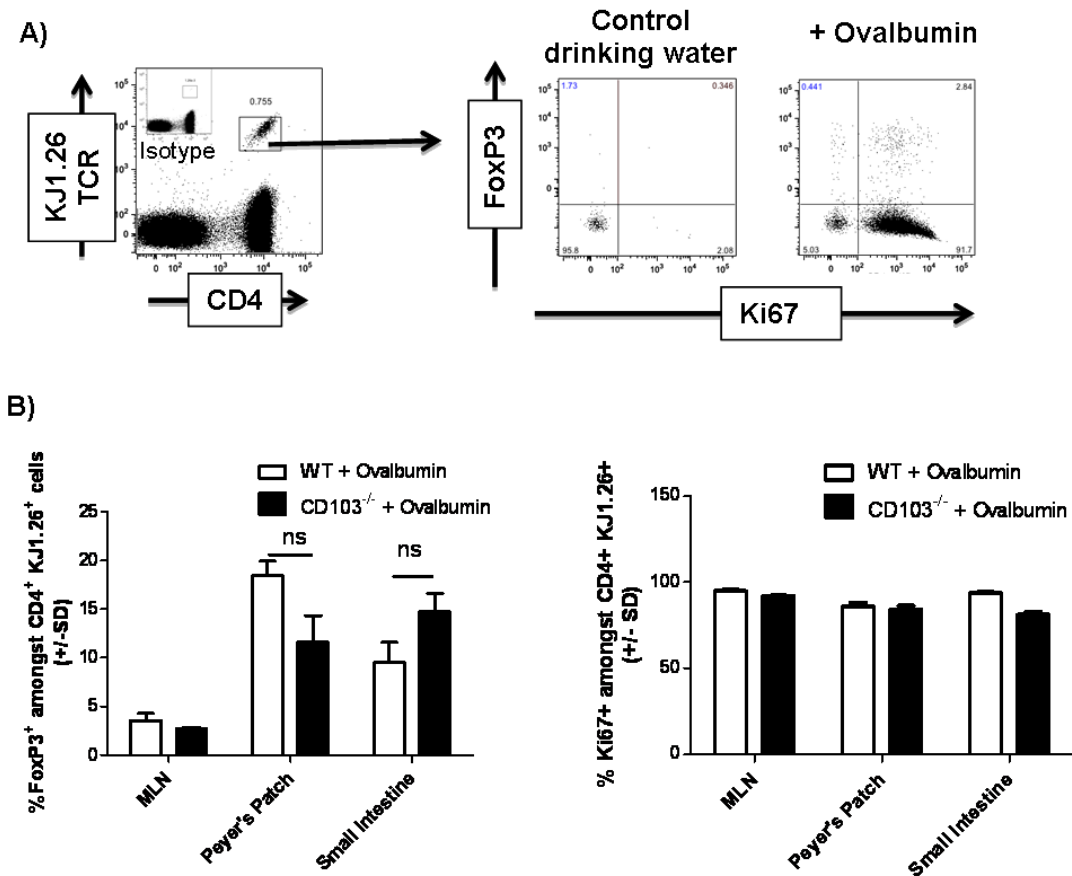


Figure 3.5 CD103 is not required for CD4⁺ T cell proliferation and FoxP3⁺ CD4⁺ T cell induction by oral antigen

Lymph node cells from DO11.10SCID mice (>95%CD4⁺ KJ1.26TCR⁺) were adoptively transferred into Balb/c wild-type (WT) mice (n=3) or Balb/c CD103^{-/-} mice (n=3). Two days following transfer mice were fed Ovalbumin (20mg/ml) in the drinking water for 5 days.

A) Identification of transferred KJ1.26⁺CD4⁺ T-cells and induction of proliferation (increase in Ki67 staining) and FoxP3 expression in response to ovalbumin

B) The relative frequency of FoxP3⁺ Tregs and Ki67⁺ cells amongst OVA-specific cells after 5 days of ovalbumin feeding.

Data represents the mean +/-SD from n = 3 biological replicates from one experiment.

Data was not significant based on a students T-test.

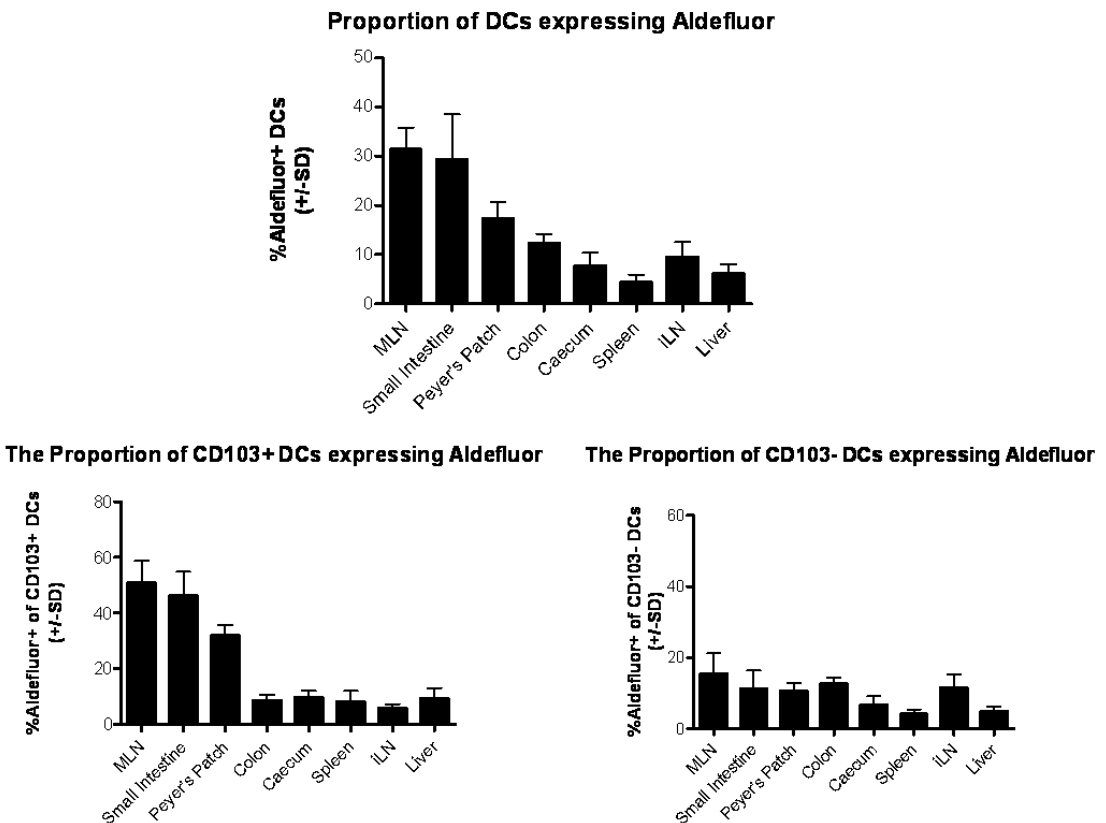
3.2.5 Tissue distribution of Aldefluor⁺ DCs *in vivo*

The indication that Aldefluor⁺ DCs in the MLN were mature and likely migratory led us to investigate the other locations *in vivo* where Aldefluor⁺ DCs could be identified. Aldefluor⁺ CD103⁺ DCs were present in the small intestine, Peyer's patches and MLN, but were largely absent from the colon, caecum or tissues more peripheral to the intestine such as the spleen, inguinal lymph node or liver (**Figure 3.6**). The absence of these cells in the large intestine was surprising and indicates an unanticipated compartmentalisation of DC phenotypes along the intestinal tract. Interestingly, a small proportion of Aldefluor⁺CD103⁻ DCs could be found in all the tissues studied, however the level of Aldefluor⁺ fluorescence produced suggests a lower level of ALDH activity and this may not necessarily reflect retinoic acid production (**Figure 3.6**).

3.2.6 ALDH activity is unaffected by antibiotic treatment or MyD88-deficiency

Data generated *in vitro* indicated that ALDH activity could be regulated by TLR ligands (see chapter 4). We therefore hypothesised that the restriction of Aldefluor⁺ DCs to the small intestine rather than the colon might be due to differences in the composition of the intestinal microbiota in the two locations. To test this we analysed the proportion of Aldefluor⁺ DCs in mice depleted of intestinal bacteria by treatment with a well established protocol of broad spectrum antibiotics [185]. In addition we analysed the proportion of Aldefluor⁺ DCs in MyD88^{-/-} mice which have a reduced ability to sense bacteria through TLR signalling. Antibiotic treatment had no effect on the proportion of Aldefluor⁺ DCs either in the intestinal lamina propria or in the MLN (**Figure 3.7A**). Similarly, there was no alteration in Aldefluor activity in MyD88-deficient mice (**Figure 3.7B**). Therefore, it is likely that ALDH activity *in vivo* is independent of the commensal flora.

A) Tissue distribution of Aldefluor⁺ DCs



B) Representative Flow Cytometry Data

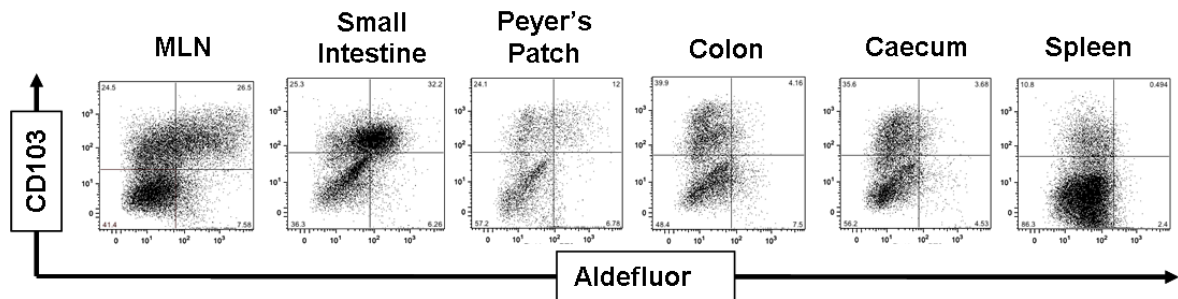


Figure 3.6 The tissue distribution of Aldefluor⁺ DCs

Single cell suspensions were prepared by collagenase digest of various organs and incubated with Aldefluor substrate prior to further incubation with antibodies to identify DCs (CD11c^{hi}MHCII⁺) by flow cytometry. Aldefluor⁺ DCs were determined relative to negative controls incubated in the presence of Deab (ALDH-inhibitor).

Data represents the mean +/-SD from n =10-12 biological replicates (MLN, Small Intestine, Colon) pooled from three independent experiments, n=5 replicates (Peyer's patch, Caecum) pooled from two independent experiments and n=3 (Spleen, iLN, Liver) from one experiment

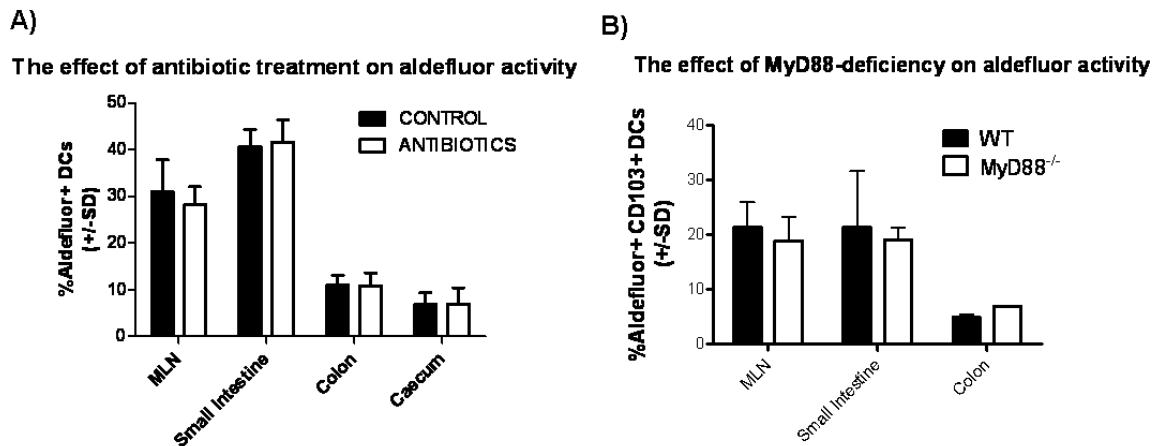


Figure 3.7 Aldefluor⁺ DCs are not controlled by the endogenous microbiota or MyD88.

Single cell suspensions were generated by collagenase digest of specified organs from mice (A) treated with broad spectrum antibiotics in drinking water for four weeks to deplete the commensal flora (n=4) or (B) MyD88-deficient mice (n=5) or WT non-antibiotic treated controls (n≥3). Cells were incubated with Aldefluor substrate and subsequently stained with fluorescent antibodies against CD11c, MHCII and CD103 to identify DCs.

Data represents the mean +/-SD from one experiment. A second experiment gave identical results.

3.2.7 Comparison of the surface phenotype of colonic and small intestinal CD103⁺ DCs

The partitioning of ALDH activity to the small intestine rather than the colon/caecum led us to further investigate the phenotype of the CD103⁺ DCs in these two locations (**Figure 3.8**). Consistent with previous reports [60, 158] a greater proportion of CD103⁺ DCs in the small intestine were CD11b⁺ than in the colon, suggesting that CD11b expression might be acquired via signals specific to the small intestinal environment. Interestingly, in contrast to lymphoid tissue DCs such as those in the spleen or MLN, a substantial proportion of CD103⁺ and CD103⁻ DCs in the lamina propria expressed F4/80, a marker more commonly associated with macrophages. This raises questions about the effectiveness of using these markers to clearly distinguish DC and macrophage populations in the lamina propria. Interestingly, a greater proportion of CD103⁺ DCs expressed F4/80 in the small intestine than in the colon and expression of F4/80 seemed to

correlate with expression of CD11b on DCs. It would be interesting to see whether the acquisition of CD11b and F4/80 by small intestinal CD103⁺ DCs is associated with any distinct functional properties, such as their localisation in the lamina propria or their phagocytic ability. Immunofluorescence studies and analysis of scavenger receptors and/or C-type lectins (many of which are also more commonly associated with macrophages) would be useful to investigate this hypothesis. Finally, the lack of expression of GR1 suggests that CD11c^{hi}MHCII^{hi} DCs are unlikely to be derived from circulating GR1^{hi} monocytes.

Analysis of the expression of co-stimulatory molecules, CD40, CD80, CD86, PDL-1 and ICOS-L, on intestinal DCs indicated that they were of a generally immature phenotype as would be expected of DCs in peripheral tissue [7]. Interestingly, PDL-1 was expressed at high levels by CD103⁻ DCs in both the colon and the small intestine although the functional relevance of this remains unclear. As PDL-1 mediated stimulation of CD4⁺ T cells has been associated with the maintenance of tolerance it may be interesting to analyse changes in PDL-1 expression resulting from inflammation or bacterial infection.

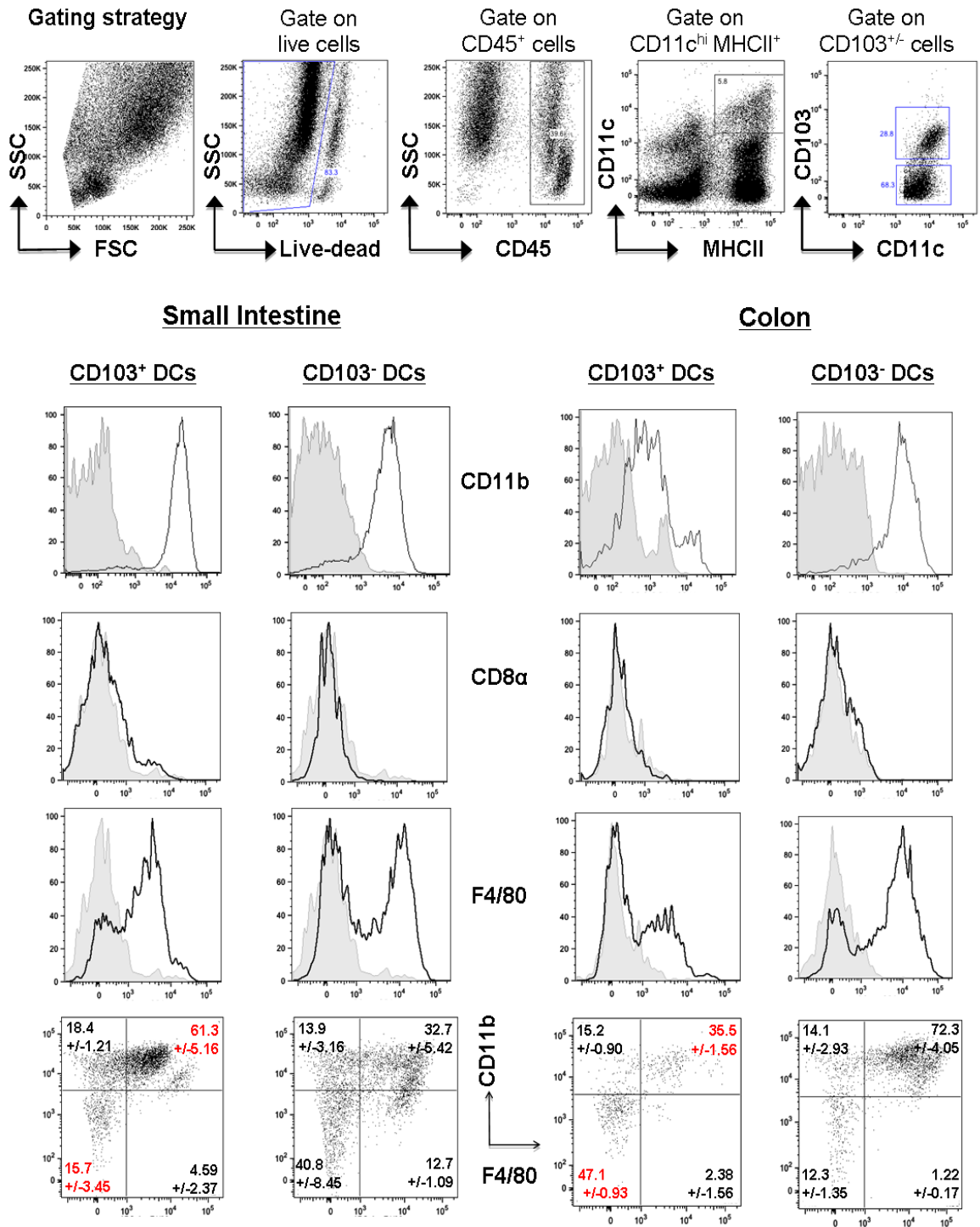


Figure 3.8 Comparison of surface phenotype of CD103⁺ and CD103⁻ DCs from the small intestine and colon

Cells were first gated as live CD45⁺CD11c^{hi}MHCII⁺ cells before analysis of various surface proteins on CD103⁺ or CD103⁻ cells. Filled histograms show fluorescence minus one (FMO) controls. The mean proportion of F4/80⁺CD11b⁺ cells amongst CD103⁺ and CD103⁻ DCs +/- SEM from n=4 biological replicates from one experiment is shown on dot-plots. Data is representative of three independent experiments for CD11b and F4/80 expression and from one experiment for CD8α expression.

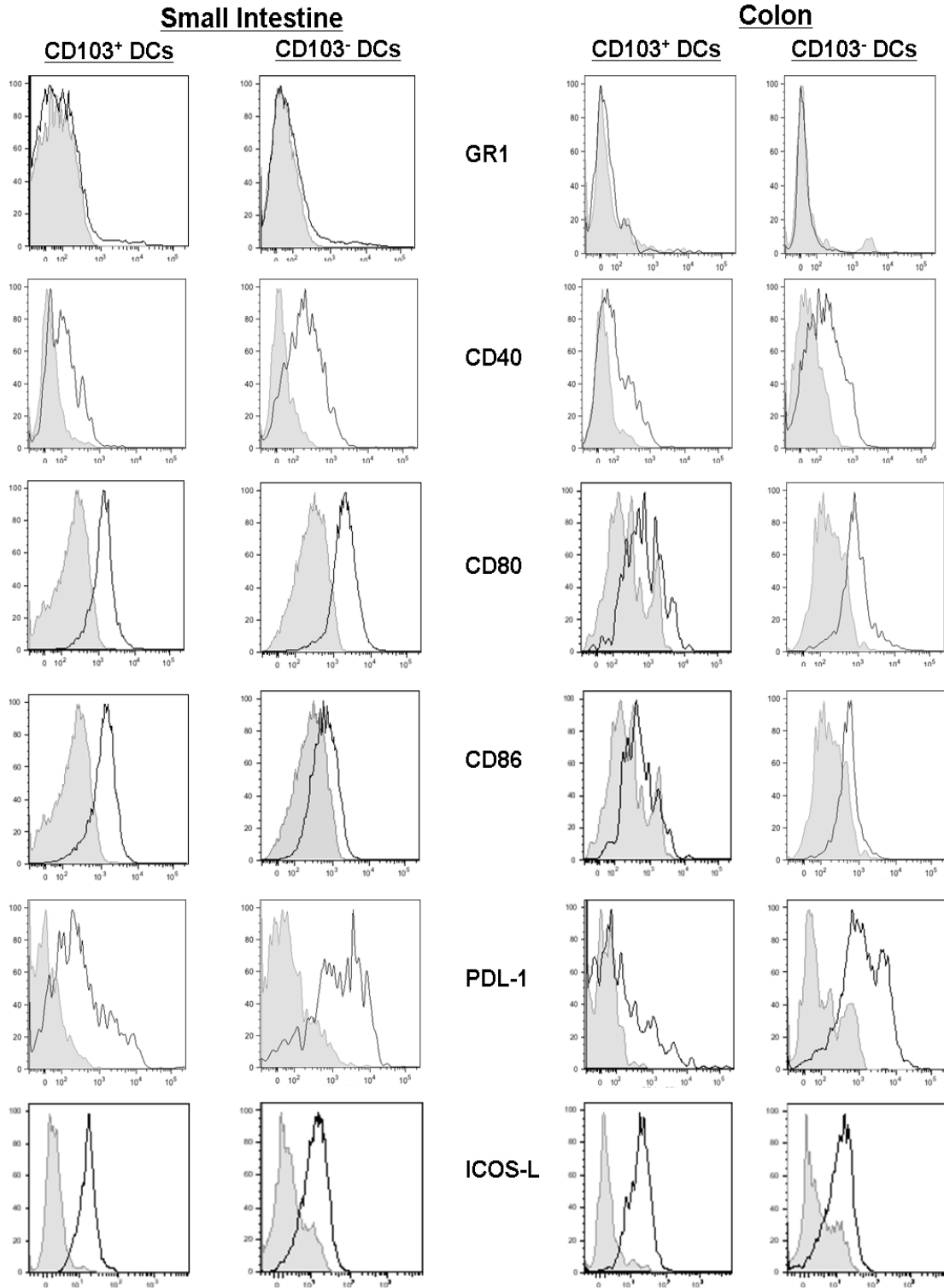


Figure 3.8 cont. Comparison of surface phenotype of CD103⁺ and CD103⁻ DCs from the small intestine and colon

Data is representative of two independent experiments for CD40, CD80 and CD86 and one experiment for PDL-1 and ICOS-L

3.2.8 An analysis of CD103⁻ DCs in the lamina propria: CX3CR1^{int} and CX3CR1^{hi} cell populations

DCs in the lamina propria have also been identified by expression of the fractalkine receptor, CX3CR1 [61, 62]. However, it was unclear how these cells related to the CD103⁺ DC subset. In order to address this, we used a CX3CR1^{GFP/+} mouse strain [61] to identify CX3CR1⁺ cells and analysed the expression of CD103 and also CD11c and MHCII. CX3CR1⁺ cells could be clearly differentiated into two populations based on the level of CX3CR1 expression both of which were CD103⁻ (**Figure 3.9A**). The first population expressed intermediate levels of CX3CR1 (termed CX3CR1^{int}) and the second expressed high levels of CX3CR1 (termed CX3CR1^{hi}) and notably these cells were also highly auto-fluorescent. CX3CR1^{int} cells showed highly variable CD11c and MHCII expression suggesting that this is a heterogeneous cell population not consisting exclusively of antigen-presenting cells. By contrast, CX3CR1^{hi} cells formed a homogenous CD11c^{lo}MHCII^{hi} population suggesting that these cells are a distinct CD103⁻ myeloid cell population likely to have antigen-presenting capability (**Figure 3.9B**).

Further analysis of surface markers on CX3CR1^{int} and CX3CR1^{hi} cells (**Figure 3.9C**) indicated that they uniformly expressed CD11b but did not express CD8 α . Substantial proportions of CX3CR1^{int} cells and all CX3CR1^{hi} cells expressed F4/80 with CX3CR1^{hi} cells expressing F4/80 at a distinctly high level (determined by MFI). This reinforces the evidence suggesting that F4/80 and CD11c are co-expressed on lamina propria antigen-presenting cell populations and therefore these markers cannot easily be used to distinguish macrophages from DCs at this location. Both CX3CR1^{int} and CX3CR1^{hi} cells expressed the co-stimulatory

molecules CD80, CD86, ICOS-L and PDL-1 raising questions about the antigen presenting and T cell stimulatory capability of these cells.

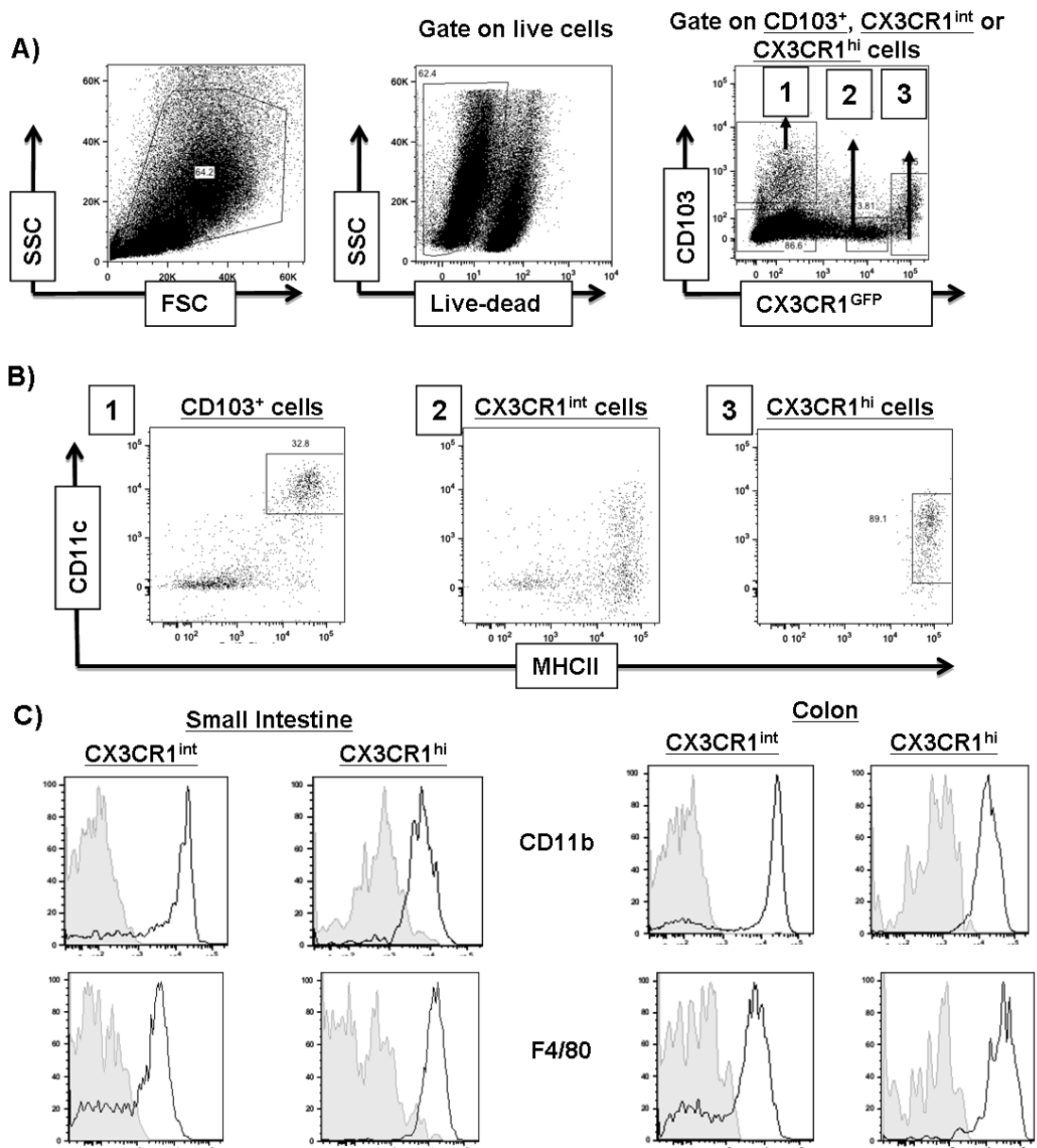


Figure 3.9 Identification and surface marker characterisation of CX3CR1^{int} and CX3CR1^{hi} cells

- A) Gating strategy to identify CD103⁺, CX3CR1^{int} and CX3CR1^{hi} cells in intestinal LPL. Data shown is from small intestine although colonic LPL shows identical profile.
- B) MHCII and CD11c profile of CD103⁺-CX3CR1⁻, CD103⁺, CX3CR1^{int}, CX3CR1^{hi} cells as gated in A.
- C) Characterisation of various surface markers on CX3CR1^{int} or CX3CR1^{hi} cells amongst leukocytes isolated from the small intestine or colon. Expression determined relative to FMO control for the channel of interest (shaded area).

Data representative of three independent experiments with n=3-4 mice per experiment

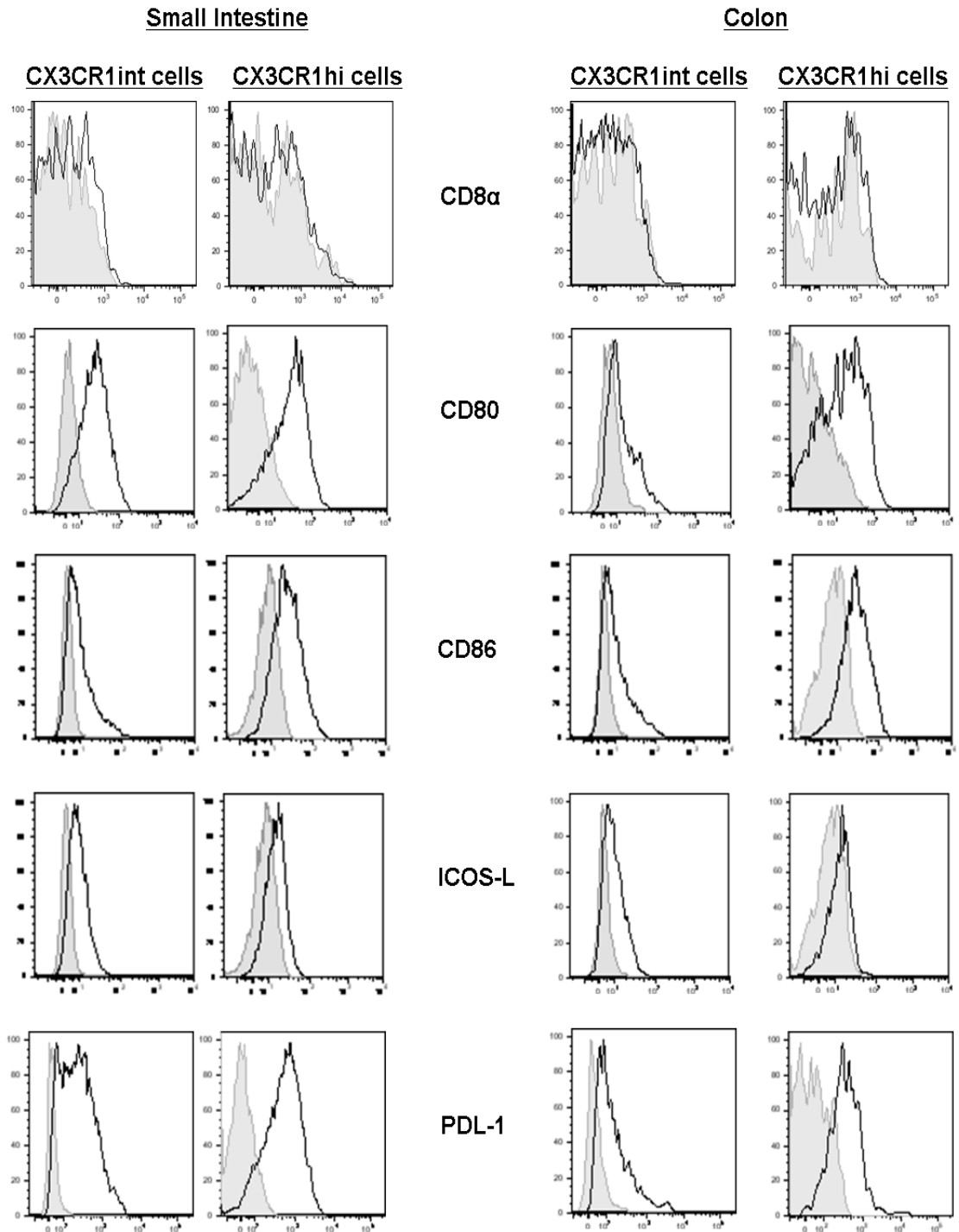


Figure 3.9 cont. Identification and surface marker characterisation of CX3CR1^{int} and CX3CR1^{hi} cells

Data representative of n=3 from one experiment

CX3CR1 has also been shown to be expressed on monocytes in the blood [34] and therefore we hypothesised that a portion of CX3CR1⁺ cells in the lamina

propria might be monocytic in origin and so would express monocyte markers, such as Ly6C. CX3CR1^{int} cells clearly contained a portion of cells which expressed Ly6C and interestingly CX3CR1^{int} Ly6C⁺ cells were CD11c⁻. This suggests a fraction of CX3CR1^{int} cells in the lamina propria are monocytes (**Figure 3.10**). As CX3CR1^{int} cells show variable CD11c and MHCII expression rather than forming distinct populations it is tempting to speculate that this population may be formed of Ly6C monocytes infiltrating the lamina propria and differentiating into antigen presenting cells.

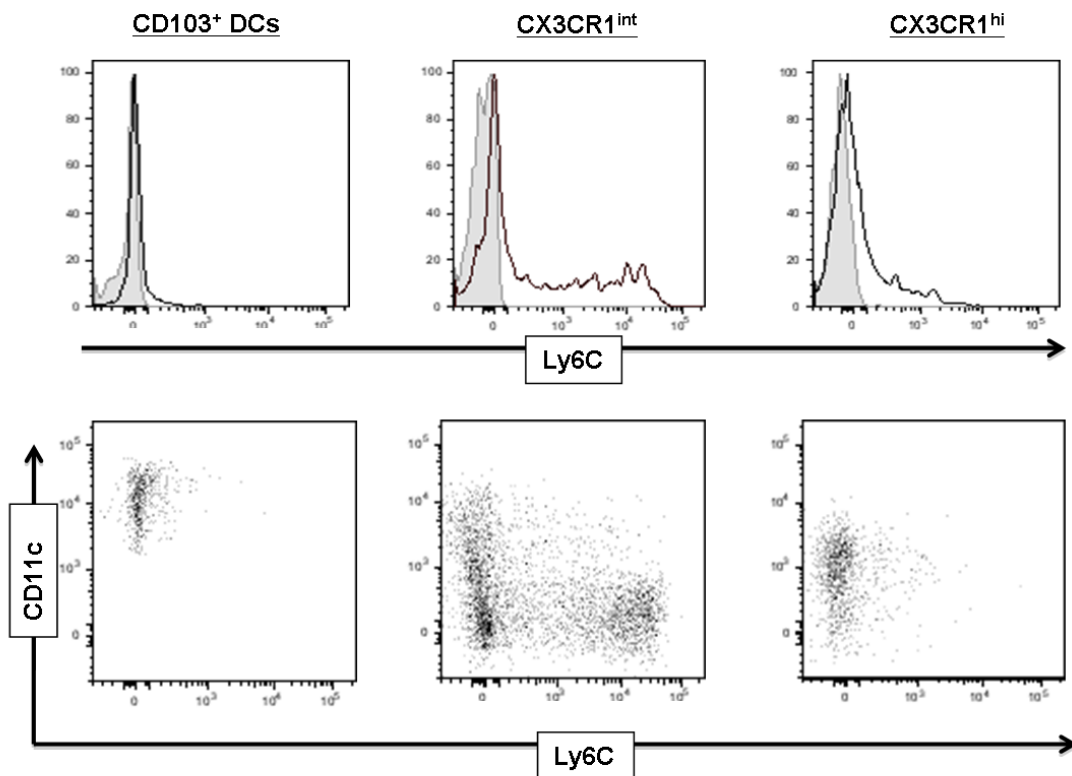


Figure 3.10 CX3CR1^{int} cells contain a population of Ly6C⁺ CD11c⁻ monocytes

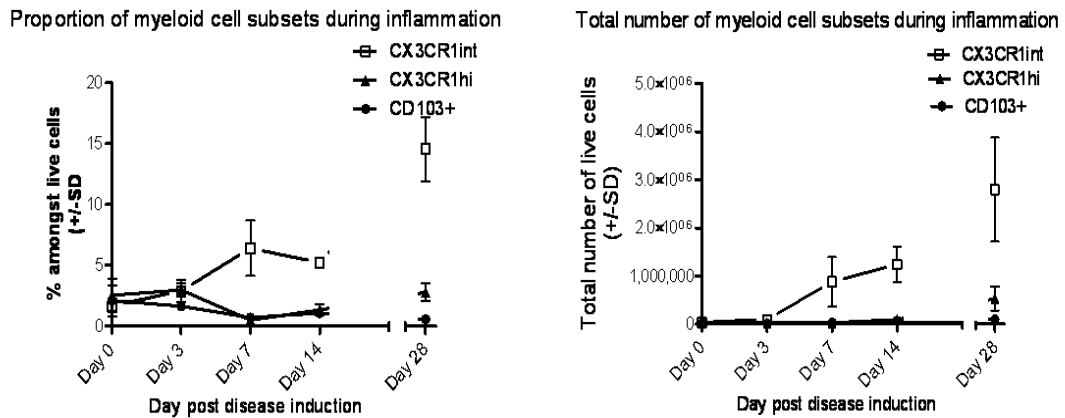
Cells extracted from the small intestine were identified by expression of CD103 and CX3CR1 respectively as shown in figure 4.9A. The profile of CX3CR1^{int} cells in the colon was similar. Expression of Ly6C was determined relative to an FMO control where all fluorescent antibodies were included except anti-Ly6C (filled histogram).

Data representative of n=6 biological replicates from two experiments.

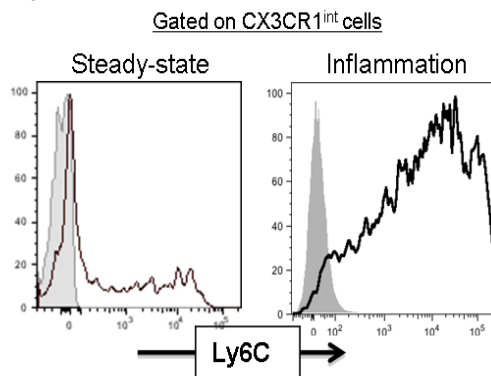
3.2.10 CX3CR1^{int} cells, but not CD103⁺ DCs or CX3CR1^{hi} cells accumulate during bacterially-induced inflammation.

The infiltration of monocyte-derived dendritic cells into inflamed tissue is one mechanism by which pathogenic infection is controlled [37, 38, 403, 404], but also by which intestinal inflammation can be exacerbated [39]. It was therefore of interest to determine whether one particular population of myeloid cells accumulated during colitis where it might be playing a more inflammatory role. We characterised the proportion and total number of CD103⁺ DCs, CX3CR1^{int} cells and CX3CR1^{hi} cells during the inflammatory responses caused by infection with the intestinal bacteria *Helicobacter hepaticus* and concurrent blockade of IL-10-signalling (with an anti-IL-10R antibody). CX3CR1^{int} cells accumulated dramatically over the course of inflammation, increasing from 3% to 15% of lamina propria leukocytes by day 28 post infection (**Figure 3.11A**). This increase in proportion translated to a substantial increase in the total number of CX3CR1^{int} cells. Furthermore, during inflammation the majority of CX3CR1^{int} cells expressed Ly6C, indicative of inflammatory monocytes (**Figure 3.11B**). There was also a proportional increase in MHCII⁺ cells amongst CX3CR1^{int} cells over the course of inflammation suggesting that infiltrating monocytes obtained an activated MHCII⁺ phenotype. However, this increase was primarily amongst CD11c⁻ cells, whereas the proportion of CD11c⁺MHCII⁺ cells amongst CX3CR1^{int} cells remained fairly constant (**Figure 3.11C**). This suggests that although there is an overall increase in the total number of CX3CR1^{int} DCs this is primarily a product of increased monocyte recruitment rather than enhancement of the differentiation of monocytes into DCs.

A)



B)



C)

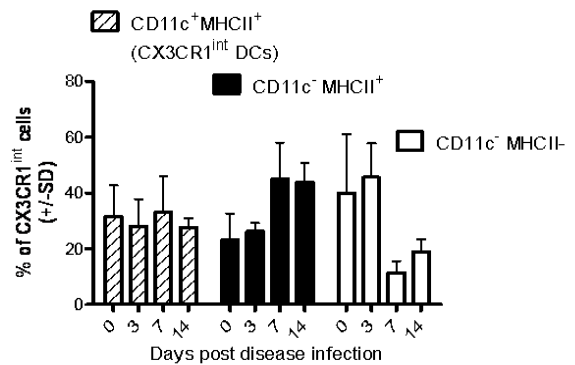


Figure 3.11 CX3CR1 intermediate cells accumulate during *Helicobacter hepaticus*- induced colitis

Mice were infected with 1×10^8 *H. hepaticus* bacteria on 3 consecutive days starting at Day 0. Mice were also injected with anti-IL-10R (1mg) on day 0 and then every 7 days while colitis develops.

A) To analyse infiltrating cells, colon LPL were isolated by collagenase digest prior to disease initiation on day 0 (n=6) and on day 3 (n=4), day 7 (n=4), day 14 (n=4) and, in a separate experiment, on day 28 (n=5). The proportions and total number of different DC subsets were determined by flow cytometry as shown previously.

B) The expression of Ly6C on infiltrating CX3CR1^{int} cells at day 28 post disease induction

C) The proportion of infiltrating CX3CR1^{int} cells that were CD11c⁺MHCII⁺ (CX3CR1^{int} DCs), CD11c⁻MHCII⁺ or CD11c⁻MHCII⁻ as determined by flow cytometry over the course of inflammation onset day 0, day3, day 7 or day 14.

Data represents the mean of n=4-6 biological replicates from one experiment. Further experiments by S. Mathisen and I. Arnold have shown similar results.

Surprisingly, over the course of inflammation there was little change in the total number of CD103⁺ DCs and instead a detectable reduction in their percentage caused by the infiltration of other cell populations. CX3CR1^{hi} cells also failed to

accumulate to a substantial level over the course of inflammation and there was a similar initial reduction in the proportion of CX3CR1^{hi} cells. However, late in disease (day 28), a small, but detectable increase in CX3CR1^{hi} cells was observed suggesting there might be some late stage enhancement of the CX3CR1^{hi} population (**Figure 3.11A**).

A preliminary analysis of cytokine production during overnight culture of colonic myeloid cell populations isolated from steady-state or from inflamed conditions was also conducted. Interestingly, for the detectable cytokines it seemed that in either the steady-state or after 28 days of inflammation the primary cytokine producers were CX3CR1^{hi} cells, with little cytokine being produced by other cell populations (**Figure 3.12**). Consistent with previous data [233], we found all subsets to be largely hyporesponsive to LPS stimulation (data not shown).

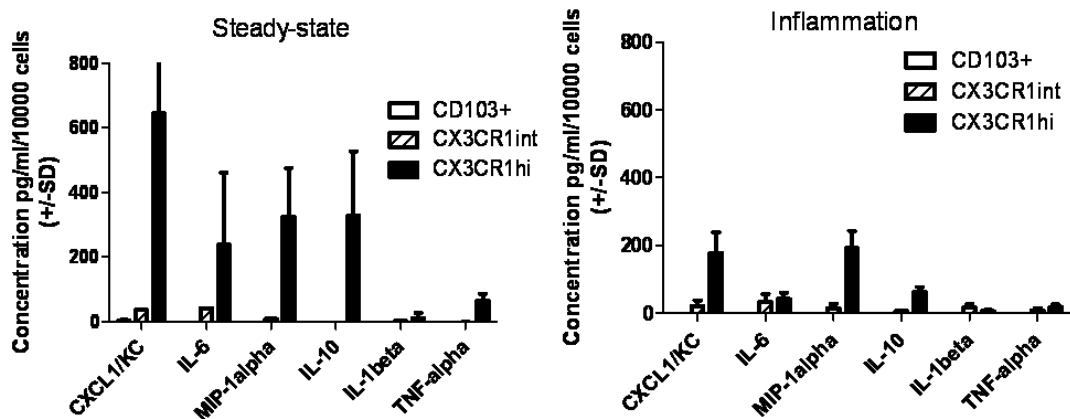


Figure 3.12 Preliminary analysis of cytokine production by intestinal DCs

In a preliminary experiment, different DC subsets were isolated to high purity by FACS from colon LPL of uninfected mice (Steady-state, n=2) or 28 days after disease induction, when colitis was evident (Inflammation, CD103⁺ n=1, CX3CR1^{int} cells (n=5), CX3CR1^{hi} cells (n=4)). Cells were cultured overnight in complete media and the concentration of cytokines secreted was measured in the supernatant by fluorescent MFI relative to standard curves (Bender Flowcytomix) and controlled to the number of cells in culture.

Data represents the mean +/- SD from one experiment

3.2.11 IL-10 production by intestinal DCs/Macrophages *in vivo* is primarily restricted F4/80^{hi} (CX3CR1^{hi}) cells

The preliminary analysis of cytokine production indicated substantial IL-10 production by CX3CR1^{hi} cells in the steady-state, but not by other lamina propria cell populations isolated (**Fig 3.12**). As IL-10 production by innate cells has a role in regulatory T cell responses [282, 311] and in reducing innate inflammation (M. Asquith, F. Powrie, K. Maloy, unpublished observations), we decided to analyse IL-10 production by intestinal DCs and macrophages *in vivo* using an IL-10^{GFP/+} reporter mouse [405].

Although, we were unable to differentiate cells directly on the basis of CX3CR1 expression in the IL-10^{GFP/+} mouse, which obviously lacks the CX3CR1^{GFP} allele, our previous characterisation of these populations indicated that it was possible to differentiate CX3CR1^{hi} cells on the basis of their extremely high F4/80 expression as all F4/80^{hi} cells expressed CX3CR1 (**Figure 3.13A**). Indeed there are clearly two populations of F4/80⁺ cells amongst lamina propria leukocytes isolated from IL-10^{GFP/+} mice. An F4/80^{int} cell population, which were CD11b⁺ and generally CD11c⁻, and an F4/80^{hi} population, which formed a homogeneous CD11c^{lo}MHCII^{hi} population identical to that observed for CX3CR1^{hi} cells (**Figure 3.13B**). The F4/80^{int} population is likely to correspond primarily to conventional macrophages in the small intestine which are generally defined as CD11c⁻CD11b⁺ [311] and a similar definition has been used to define macrophages in the colon [406]. However, more recent data suggests F4/80 also identifies populations of eosinophils in the colon, which may explain why many of these F4/80^{int} cells are MHCII⁻ and despite attempts to gate eosinophils out by Forward and Side-Scatter we can not rule their presence out entirely [310, 407, 408]. Therefore, we

identified F4/80^{hi} cells (equivalent to CX3CR1^{hi} cells), F4/80^{int} cells (representative of conventional macrophages/eosinophils), CD103⁺ DCs and CD103⁻ DCs in IL-10^{GFP/+} mice strain and determined the proportion of each population that were expressing IL-10^{GFP} relative to an IL-10^{+/+} (i.e. non-GFP) control.

Consistent with cytokine production by lamina propria cells *in vitro* (**Figure 3.13**), IL-10^{GFP} expression was detectable primarily amongst F4/80^{hi} cells (**Figure 3.14**). Although the fluorescent signal was extremely low as has been observed for innate cells in this strain previously [405] it represented a shift of the whole population, was observed in both the colon and small intestine and was highly reproducible across three separate experiments each with >4 biological replicates. By contrast, few IL-10^{GFP} cells could be detected amongst the CD103⁺DCs, CD103⁻DCs and F4/80^{int} cells (macrophages/eosinophils) and these represented fewer than 5% of each respective cell population (**Figure 3.13 and Figure 3.14A**). Therefore, it is apparent that F4/80^{hi} (CX3CR1^{hi}) cells are the primary source of myeloid cell-derived IL-10 in the intestine. In addition, we were unable to detect IL-10^{GFP} expression from other innate cell types such as innate lymphoid cells (data not shown) suggesting that the primary innate source of IL-10 is the F4/80^{hi} cell population.

Given the extremely low level of GFP-fluorescence detectable in these populations it is important to reproduce this result using other IL-10-reporter strains including one recently described to be more sensitive for innate IL-10 production [409]. In addition, it would be beneficial to validate the ability to detect innate IL-10 using this strain by stimulating splenocytes with IL-10-inducing ligands such as zymosan *in vitro*. Finally it may be possible to amplify the GFP signal using anti-GFP antibodies.

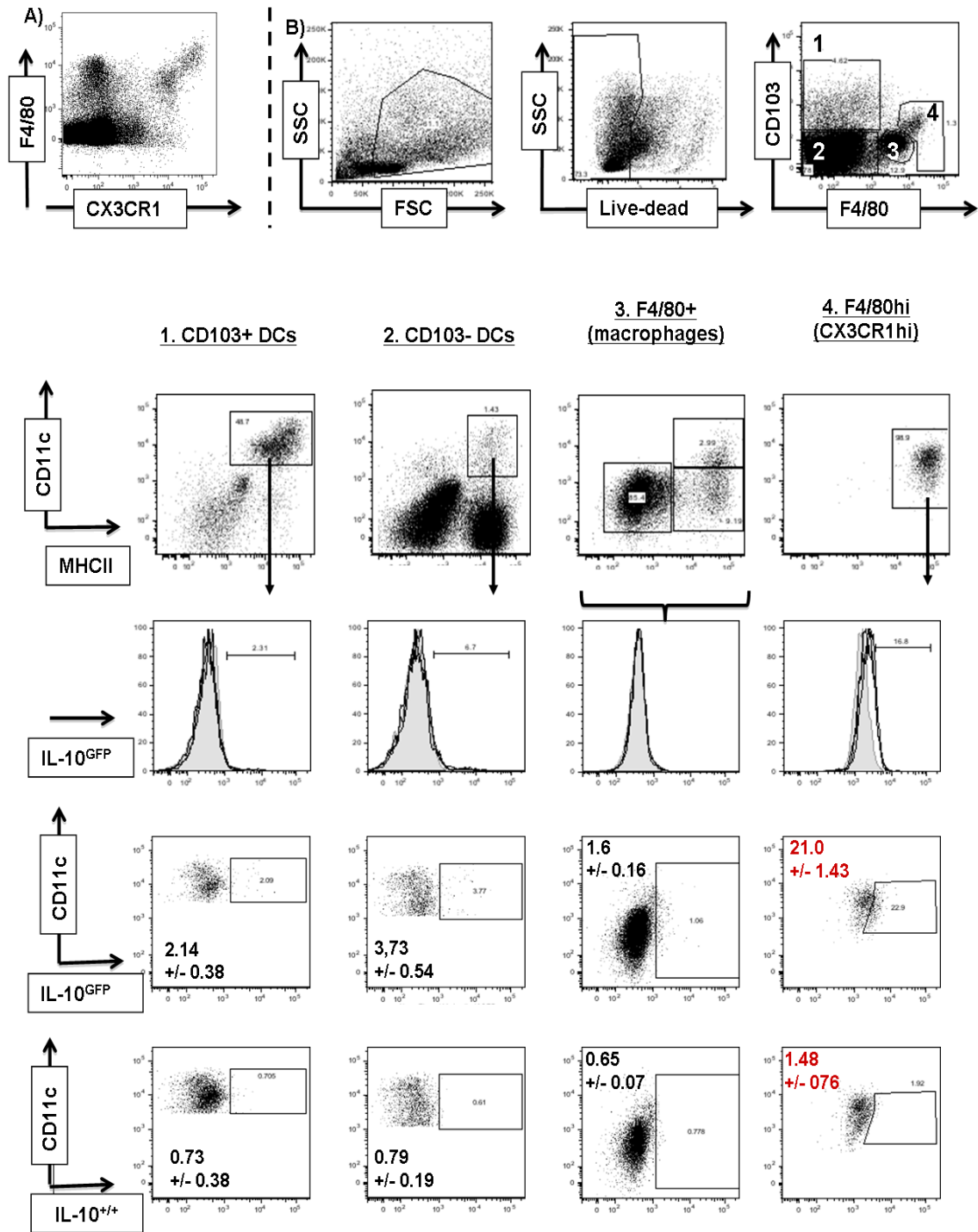


Figure 3.13 F4/80^{hi} cells, which overlap with CX3CR1^{hi} cells, uniquely express IL-10 in the steady-state

A) CX3CR1^{hi} cells can be identified by F4/80 expression.

B) Cell suspensions were generated by collagenase digest of colon from IL-10^{GFP/+} mice (solid lines on histogram and row 4) or IL-10^{+/+} mice (shaded area on histograms and row five). Populations of DCs/macrophages were identified by expression of CD103, F4/80, CD11c and MHCII using fluorescent antibodies relative to FMO controls and expression of IL-10^{GFP} assessed.

Data representative of 6 biological replicates from two independent experiments. Proportions IL-10^{GFP+} cells amongst respective cell populations are quantified on dot plots +/- SD and in fig 3.14

3.2.12 Innate IL-10 is not enhanced by *Helicobacter hepaticus* infection or controlled by MyD88-signalling in the steady-state.

The ability to tolerate the intestinal bacteria *Helicobacter hepaticus* relies on the expression of IL-10 [278]. We therefore hypothesised that infection with *H. hepaticus* may enhance the low level of IL-10^{GFP} expression detected in intestinal DCs/macrophages. One week after infection with *H. hepaticus* there was no detectable increase in IL-10^{GFP} expression by F4/80^{hi} cells, which produce most IL-10 in uninfected animals. Although IL-10^{GFP} expression remained at very low levels for macrophages/eosinophils, CD103⁺ DCs and CD103⁻ DCs, a small, but a significant increase was detectable after infection in the colonic CD103⁻ DC population and the small intestinal CD103⁺ DC population (**Figure 3.14A**).

In order to try and determine the mechanism by which IL-10 is regulated in myeloid cells we analysed the IL-10^{GFP} expression in IL-10^{GFP/GFP}MyD88^{-/-} mice which are reduced in their ability to respond to bacterial products through Toll-like receptors. Surprisingly, we found that IL-10^{GFP} expression in F4/80^{hi} cells, CD103⁺ DCs and CD103⁻ DCs was not dependent on MyD88 (**Figure 3.14B**). Interestingly, however, the small proportion of colonic macrophages/eosinophils which expressed IL-10^{GFP} were absent in the colon of MyD88^{-/-} mice, consistent with previous reports showing that expression of IL-10 mRNA in isolated colonic macrophages was reduced in MyD88-deficient mice [406].

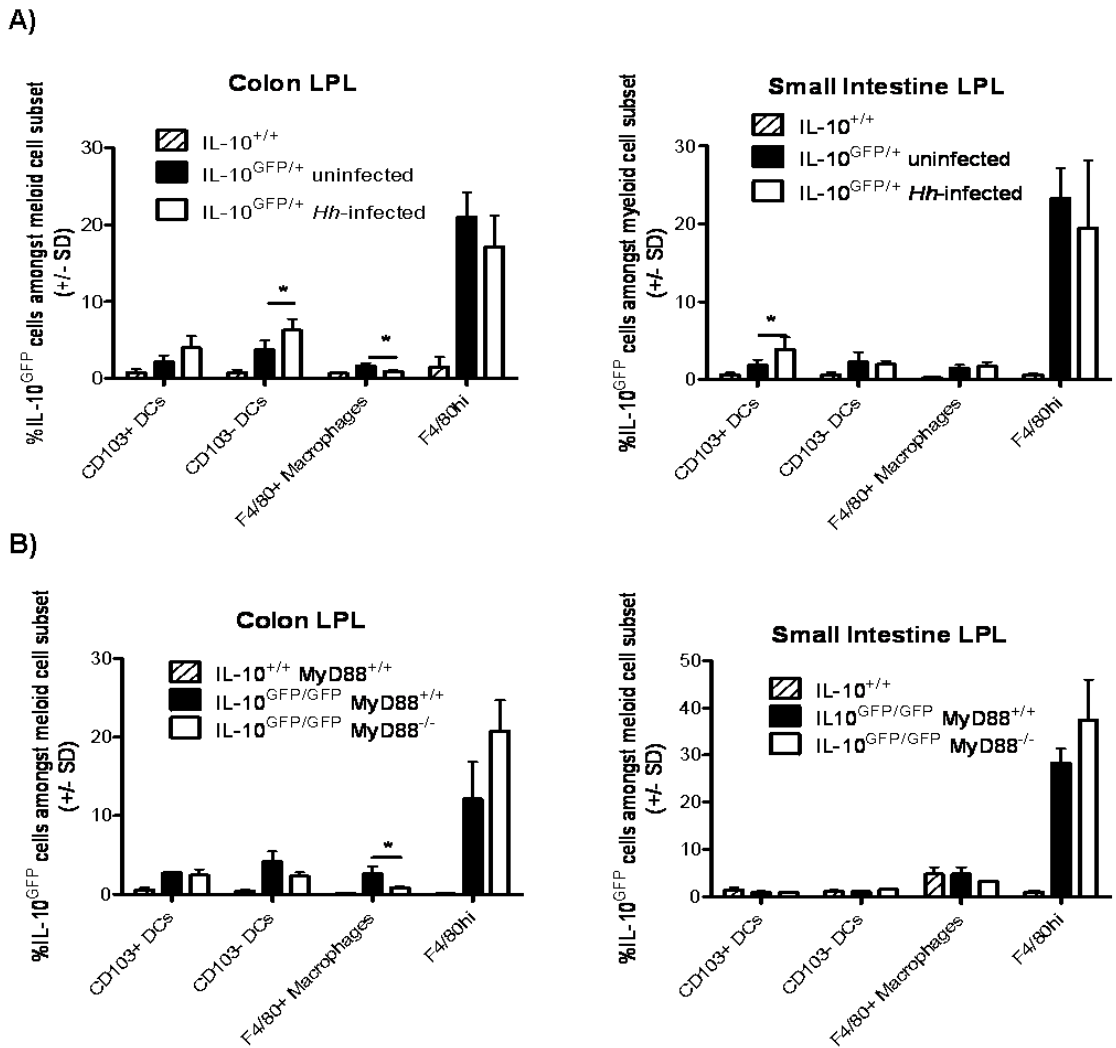


Figure 3.14 The control of IL-10 expression in intestinal myeloid cells

- A) In order to determine whether IL-10 was regulated by *Helicobacter hepaticus* IL-10^{GFP/+} mice were infected with 1×10^8 *Helicobacter hepaticus* on three consecutive days. Seven days later cell suspensions were obtained by collagenase digest of the colon or small intestine and the proportions of IL-10^{GFP} cells amongst DC/macrophage populations was determined by co-staining with CD103, F4/80, CD11c and MHCII as shown in figure 4.14.
- B) In order to determine whether IL-10 was regulated by MyD88, IL-10^{GFP/GFP} were crossed to MyD88^{-/-} mice (M. Asquith, M. Barnes, unpublished data). The proportions of IL-10^{GFP} cells amongst DC/macrophage populations was determine by flow cytometry.

In both cases data represents the mean +/-SD from n=3-5 biological replicates from one experiment

Statistical significance was determined using a Mann Whitney test (*p<0.05)

3.3 Discussion

Dendritic cells *in vivo* form a diverse network, consisting of distinct subsets, variable maturation states and specific anatomical distributions. All of these combine to provide a mechanism to resist infection while avoiding immune responses against harmless stimuli. The full extent and complexity of this network at mucosal sites is only recently becoming apparent and much has relied on the improvement in cell isolation techniques from such tissues [410, 411]. Indeed a considerable amount of the work presented here identifying DC populations in the intestine was dependent on the integration and improvement of published protocols to extract leukocytes from the non-inflamed intestinal lamina propria. However, as noted by Pabst and Bernhardt, the numbers of DCs obtainable from the lamina propria remain low and highly variable compared to the levels estimated from immunofluorescent microscopy of intestinal tissues [411]. Therefore, isolation methods remain sub-optimal confounding the interpretation of results. Nevertheless, through many recent studies investigating DC subsets in the intestine [24, 25, 35, 310, 412, 413], of which this thesis represents a small part, we can build a reasonable picture of the layout of the myeloid cell network in the GALT and intestinal lamina propria. A description of this network, together with outstanding questions, is discussed below.

3.3.1. The myeloid cell network in the intestine

In this chapter we have shown that two different populations of CD103⁺ DCs populate the intestinal lamina propria; CD103⁺CD11b⁺F4/80⁺ALDH⁺ DCs (henceforth referred to as CD103⁺CD11b⁺ DCs) which are enriched in the small intestine and CD103⁺CD11b⁻F4/80⁻ALDH⁻ DCs (henceforth referred to as CD103⁺CD11b⁻ DCs) which are enriched in the colon. In addition to these

populations we also describe two populations of CX3CR1⁺ cells; CX3CR1^{int} cells and CX3CR1^{hi} cells which are both present in approximately equal proportions in the small intestine and colon. CX3CR1^{int} cells were highly heterogeneous including some Ly6C⁺ cells likely to be monocytes. CX3CR1^{hi} cells were homogeneously F4/80^{hi}CD11c^{lo}MHCII^{hi}. The phenotypes of these four myeloid cell populations present in the lamina propria is summarised in **figure 3.15**.

The fact that F4/80, CD11c, MHCII and various co-stimulatory molecules could be found on all these myeloid cell populations shows that these classical “DC” and “macrophage” markers should be used with caution when identifying myeloid cells in the lamina propria. These findings are in agreement with recently published data which have also analysed the development and migratory capacity of these populations [24, 35, 410]. Interestingly, both CD103⁺ DC populations derive from pre-DCs in response to FLT3L, aligning them to the conventional DC lineage. However, CX3CR1^{hi} cells and to some extent CX3CR1^{int} cells derive from monocytes in response to M-CSF, perhaps aligning them more closely to a macrophage lineage (**see figure 1.2**). As shown here CX3CR1^{int} cells are a highly heterogeneous cell population and it is apparent that to some extent this population can also derive from pre-DCs [25]. Further work determining the precise phenotype of CX3CR1^{int} cells derived from monocytes or pre-DCs may help to further define this population. CX3CR1⁺CD11c⁺ cells have been previously described to extend dendrites through the lamina propria as part of an antigen sampling mechanism [62]. It is not clear whether these represent CX3CR1^{hi} cells or a subset of CX3CR1^{int} cells. We have been unable to identify either population amongst leukocytes isolated from the epithelial layer suggesting that they mostly remain embedded in the lamina propria.

The distinct nature of CD103⁺ CD11b⁺ and CD103⁺ CD11b⁻ DC subsets is further reinforced by analysis of their ontogeny. CD103⁺CD11b⁺ DC development is partially GM-CSF dependent [24] whereas CD103⁺CD11b⁻ DCs develop independently of GM-CSF. This is discussed further in chapter 4. Furthermore, whereas CD103⁺ CD11b⁻ DCs are dependent on the transcription factors Batf3, IRF8 and ID2, CD11b⁺CD103⁺ DCs are independent of these factors [414, 415]. Batf3 is also required for CD8 α ⁺CD11b⁻ DC development in lymphoid tissues [51]. Therefore, peripheral CD103⁺ CD11b⁻ DCs, which interestingly we did not find to express CD8 α themselves, are in fact related to the CD8 α ⁺CD11b⁻ DC lineage. This raises the suggestion that CD103⁺CD11b⁻ DCs could be restricted to lymphoid tissue in the intestines whilst CD103⁺ CD11b⁺ DCs could be dispersed throughout the lamina propria. This hypothesis is supported by previous histological studies, using a limited panel of markers, which suggested that colonic DCs are primarily located in lymphoid follicles whereas small intestinal DCs are scattered throughout the lamina propria [312]. Furthermore, CD103⁺ CD11b⁻ DCs are not found in the small intestine of ID2^{-/-} mice, which lack the ability to form Peyer's patches, supporting the suggestion that these cells are restricted to lymphoid tissue [24]. Lymphoid tissue in the small intestine takes the form of Peyer's patches which can be easily excised. However, it is not possible to remove isolated lymphoid follicles in the colon. The removal of Peyer's patches from the small intestine in our protocol may thus explain why CD103⁺CD11b⁺ DCs are comparatively enriched in this location. Detailed immunofluorescent analysis to determine the localisation of DC subsets in the colonic and small intestinal lamina propria is currently being undertaken.

We show here that colonic and small intestinal CD103⁺ DCs can also be distinguished based on their ALDH activity. This is consistent with recently published data and previous indications that colonic CD103⁺ DCs were unable to induce the expression of gut homing receptors on T cells *in vitro* [396, 416]. Indeed, recent data has shown that CD103⁺ CD11b⁻ DCs from other locations *in vivo* such as the skin and the lung are also ALDH⁻ [401]. This is probably due in part to GM-CSF signalling in the small intestine as ALDH activity is ablated in small intestinal DCs from GM-CSFR^{-/-} mice [341]. However, recent data has also shown that vitamin A (metabolised to retinoic acid *in vivo*) enhances ALDH activity [341, 416-418]. This is partly mediated by dietary derived vitamin A and by retinoids secreted by the liver into the bile [416] and partly due to retinoic acid signaling in the bone marrow which targets DCs to the small intestine via the induction of CCR9 expression [417]. Whether vitamin A and its derivatives also induce other aspects of the CD103⁺CD11b⁺ DC phenotype, such as β 8-expression, remain unclear. A recent study has shown that a β -catenin mediates program of gene expression in DCs which is essential for the maintenance of tolerance in the intestine [389]. It will be interesting to determine whether retinoic acid signaling can activate β -catenin and thus induce a wider program of DC conditioning.

3.3.2 Functional features of intestinal myeloid cells

The extremely low cell numbers obtainable from the intestinal lamina propria and the extensive manipulation required to extract them severely limits the ability to undertake functional analysis of intestinal myeloid cell populations. Indeed, we have been frustrated not to have been able to take this study far beyond the descriptive stage. However, our preliminary data and other recently published

studies have begun to indicate functional features of these various myeloid cell populations.

CD103⁺ CD11b⁺ (ALDH⁺) DCs

Elegant intra vital microscopy and flow cytometric analysis of intestinal lymph has indicated that only CD103⁺ DCs migrate from the intestine to the MLN whereas CX3CR1⁺ cells remain resident in the lamina propria [35]. This supported previous data indicating that CD103⁺ DCs are likely to be migratory [24, 157, 323]. Our data show that all ALDH⁺ DCs, determined by Aldefluor reactivity, in the MLN are highly mature and CCR7⁺. This suggests that small intestinal derived CD103⁺ CD11b⁺ DCs are likely to be migratory, supporting data showing these cells carry *Salmonella* to the MLN following oral infection [24]. However, it is not clear whether CD103⁺ CD11b⁻ DCs from the colon also migrate in this fashion. The presence of CD103⁺ ALDH⁻CCR7⁺ DCs in the MLN might suggest that they do, but further work attempting distinguish and track migration of colonic DCs is required to confirm this.

Our data and others indicate that ALDH-activity correlates with the expression of the gene ALDH1A2, which is highly expressed in small intestinal and MLN CD103⁺ DCs and likely determines their ability to produce retinoic acid [157, 338, 341]. Retinoic acid production underlies the ability of small intestinal and MLN DCs to induce gut homing receptors, IgA class switching and together with TGF- β enhances FoxP3⁺ Treg cell induction during homeostasis [156-158, 323, 327, 335, 337-339]. We also show here that expression of the TGF- β activating integrin, β 8, which is required for oral tolerance, is enriched amongst CD103⁺ ALDH⁺ DCs in the MLN compared to ALDH⁻ DCs. Furthermore, despite expressing high levels of co-stimulatory molecules thought to inhibit iTreg cell differentiation [156],

CD103⁺ALDH⁺ DCs are able to preferentially promote iTreg cell differentiation *in vitro*. Therefore, our data suggest that CD103⁺ALDH⁺ DCs do act in a more “tolerogenic” manner during homeostasis. This is consistent with recent data showing β 8-integrin to be expressed on MLN CD103⁺ DCs and to be critical for iTreg induction in response to oral antigen [419].

Although retinoic acid production is a distinctive feature of CD103⁺ DCs, it is by no means restricted to these cells alone within the intestine. ALDH1A enzymes are expressed at high levels by epithelial cells in the intestine [338, 343, 420-422] and also by stromal cells within the MLN [342]. Consequently, these cells are also capable of inducing the expression of gut-homing chemokine receptors such as CCR9 [342]. Nevertheless, it is conceivable that retinoic acid production by DCs might have non-redundant consequences for the adaptive immune response. Mice expressing LoxP-flanked ALDH1A2 alleles have been generated [423] and the generation of a DC-specific (CD11cCre) ALDH1A2^{-/-} and subsequent analysis may reveal non-redundant functions for DC-derived retinoic acid.

CD103⁺ CD11b⁻ (ALDH⁻) DCs

In the intestine the functional features of CD103⁺CD11b⁻ DCs remain unknown and studies in Batf3^{-/-} mice indicated little role for this subset in regulating susceptibility to DSS-induced colitis or in iTreg cell induction in response to oral antigen. The close relationship of CD103⁺ CD11b⁻ DCs to CD8 α ⁺ DCs suggests that these cells may have a role in cross-presentation of antigen and the polarisation of Th1 responses. Indeed, analogous populations of DCs in the skin and the lung have recently been shown to efficiently cross-present apoptotic cell-derived, viral and self antigen respectively [424, 425]. Therefore, CD103⁺CD11b⁻ DCs may be specialised to maintain cross-tolerance during homeostasis and

perhaps fight infection. Indeed it has recently been shown that $Batf3^{-/-}$ mice are deficient in their ability to resist *Toxoplasma Gondii* infection [426]. We are conducting a microarray of $CD103^{+}CD11b^{-}$ DCs in order to define more clearly the specific features of this population which may play functional roles.

CX3CR1^{int} cells

We show here that CX3CR1^{int} cells accumulate dramatically during bacterially-induced inflammation consistent with recent reports in the T cell transfer and DSS-colitis models [413, 427]. In this lab, Siddiqui *et al* had previously shown the accumulation of monocyte-derived DCs during colitis which stained positive with an anti-E-Cadherin antibody [39]. Preliminary data strongly suggests these overlap with the CX3CR1^{int} cell population that we show accumulating here (Stephanie Mathisen, A. Johnson, F. Powrie, unpublished data). The accumulation of CX3CR1^{int} cells implicates them in perpetuating inflammation and how they function in this setting is an area of active research. In the preliminary experiment presented here, we detected only limited cytokine production from CX3CR1^{int} DCs isolated from inflamed colons after four weeks of disease. This time-point for analysis was consistent with histological markers of disease progression [198], however further analysis of cellular changes, including characterisation of CD4⁺ T cell responses, suggests that the inflammatory response may peak 2-3 weeks post disease induction. Therefore, the analysis of cytokine production by CX3CR1^{int} cells at earlier time points may be optimal to detect cytokine production (M. Shale and A. Johnson, unpublished observations).

CX3CR1^{hi} cells

The exacerbated DSS-colitis that ensues in DC-depleted mice reconstituted by CX3CR1^{hi} cells alone was interpreted to indicate CX3CR1^{hi} cells are more pro-inflammatory in phenotype [25]. In fact, this phenotype could equally be caused by a loss of CD103⁺ DC-mediated control. Other recent data has implicated CX3CR1^{hi} cells and overlapping populations of “macrophages” (based on CD11b, MHCII and F4/80 expression) in the production of IL-10 and the IL-10-dependent induction of FoxP3⁺ CD4⁺ T cells in the lamina propria [282, 308, 311]. Although CD4⁺ T cell-derived IL-10 has a dominant function in preventing tolerance [280, 288], innate cells can have significant supplementary roles [281, 282] (M. Asquith, K. Maloy, F. Powrie, unpublished data). The data presented here analysing the expression of IL-10^{GFP} in intestinal myeloid cells and the production of IL-10 by isolated myeloid cell populations *in vitro* shows that IL-10 production is restricted to CX3CR1^{hi} (F4/80^{hi}) cells. Interestingly, the bulk population of F4/80⁺CD11c⁻ macrophages were largely IL-10^{GFP-}. However, consistent with recent reports analysing IL-10 mRNA level in this cell population, all IL-10^{GFP+} macrophages that we did detect were absent in MyD88^{-/-} mice [406]. As shown here and by others, the fluorescent signal of IL-10^{GFP} in myeloid cells within this reporter mouse strain is extremely low [405]. This could reflect a genuinely low level of IL-10 transcription in myeloid cells or technical concerns about the handling of GFP and the high level of background fluorescence displayed by these cell types. It is reassuring that other groups have found similar results using a different IL-10 reporter (IL-10^{vert-x}) [428], however a recently described, more sensitive, IL-10 reporter strain would be useful to further study the regulation of IL-10 in CX3CR1^{hi} cells [409].

We also show here that CX3CR1^{hi} cells express high levels of MHCII and detectable levels of many co-stimulatory molecules again consistent with recently published data [25, 35]. This is despite the fact there is little evidence that these cells migrate to lymph nodes to present antigen to naive T cells [35]. This raises questions about the role of CX3CR1⁺ cells in the presentation of antigen within the lamina propria perhaps as a form of restimulation or “in-tissue licensing” as has recently been described for Langerhans Cells in the skin [100]. The development of strategies to selectively deplete CX3CR1^{hi} cells would allow analysis of the pro- or anti-inflammatory nature of these cells and their antigen-presenting capacity to be assessed *in vivo*. This strategy for studying DC function *in vivo* is further discussed in sections 6.2 and 6.3 in the context of an integrated view of the myeloid cell network and future experiments.

Chapter 4

The differentiation of CD103⁺ALDH⁺ dendritic cells *in vitro*

4.1 Introduction

The small proportion of DCs found *in vivo* and the technical difficulty of extracting them makes *in vitro* culture an important tool for studying and manipulating DC function. Furthermore, understanding which precursors and growth factors are required for the ontogeny of a DC subset is important when considering the basic underlying phenotype and a particular subset's relationship to other myeloid cell populations. We therefore undertook to study the differentiation of CD103⁺ DCs *in vitro* and used this system to study the factors important in promoting ALDH activity, a key functional feature of intestinal CD103⁺ DCs.

Extensive and detailed work over the past decade and more has described the pathway of DC ontogeny *in vivo*. Two growth factors, in particular, have been highlighted, FLT3L and GM-CSF, which can be used to generate DCs from bone marrow progenitors *in vitro* and to expand DCs *in vivo* [390, 391, 429-431][141, 432, 433]. FLT3L administration results in expansion of all lymphoid tissue DC subsets and complementally FLT3^{-/-} and FLT3L^{-/-} mice have substantial defects in lymphoid tissue DC numbers clearly showing the importance of FLT3L to steady-state DC development [28, 430, 431]. Conversely, GM-CSF administration appears to expand only the CD11b expressing, previously termed "myeloid", DC populations [141]. Furthermore, GM-CSF^{-/-} mice had only minor defects in lymphoid tissue DC numbers, and there were only minimal additional reductions in lymphoid tissue DC numbers in FLT3L^{-/-}GM-CSF^{-/-} double knock-out mice compared to FLT3L^{-/-} alone. Therefore, GM-CSF has little role in controlling the

steady-state development of lymphoid tissue DCs [26, 28, 430, 434]. Instead, analysis of non-lymphoid tissue including mucosal sites such as the intestine has suggested that GM-CSF appears more important in driving monocyte-derived DC accumulation [24, 25, 33, 36, 39, 435]. However, such monocytic contribution to the DC pool, even in non lymphoid tissue, remains highly contentious and appears likely to be relevant primarily during inflammatory responses and less so under homeostatic conditions. Consistent with this, GM-CSF-driven BMDCs have a phenotype equivalent to TiP-DCs [436].

We therefore sought to analyse the development of CD103⁺ DCs in BMDC cultures supplemented with either GM-CSF or FLT3L or following emerging data, a combination of the two. We then used this system to begin to analyse the induction of an intestinal CD103⁺ DC-like phenotype, including ALDH activity, which is a sign of the ability to produce retinoic acid, and the ability of these DCs to induce FoxP3⁺ Treg cells.

4.2 Results

4.2.1 CD103⁺ and E-Cadherin⁺ DC differentiation in response to FLT3L or GM-CSF.

Despite the clear generation of DCs in bone-marrow cultures supplemented with GM-CSF or FLT3L alone (>90% of cells in culture CD11c⁺ MHCII⁺), only a small proportion of these expressed CD103 (~10%, **Figure 4.1**). Interestingly a high proportion of DCs induced by GM-CSF expressed E-Cadherin (~50%) a marker of monocyte-derived inflammatory DCs that accumulate during intestinal inflammation [39]. By contrast, such accumulation of E-Cadherin⁺ DCs was not

observed in cultures induced by FLT3L (**Figure 4.1**), consistent with GM-CSF alone driving a monocyte-derived DC lineage.

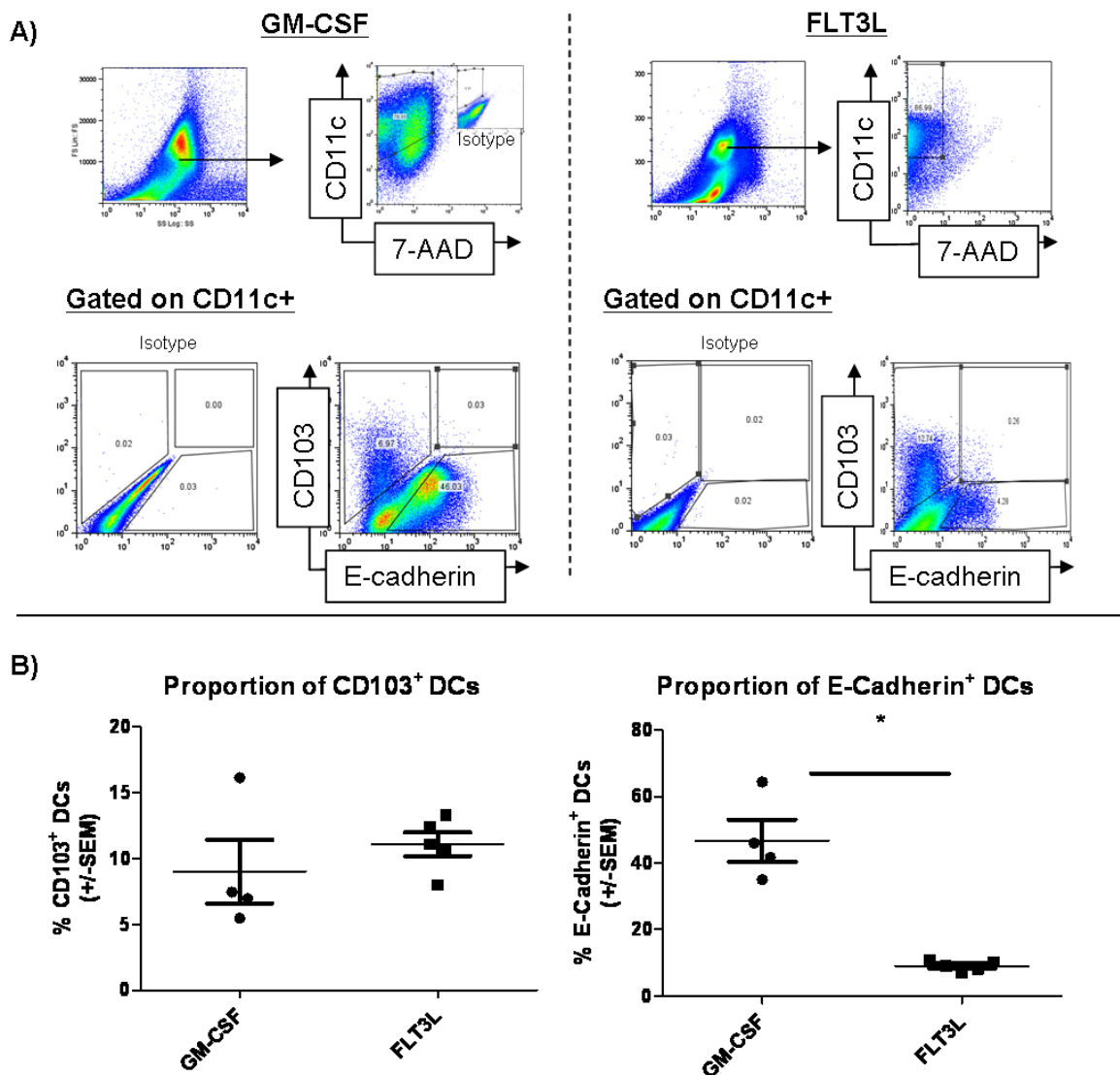


Figure 4.1 The generation of CD103⁺ DCs and E-Cadherin⁺ DCs in GM-CSF or FLT3L-differentiated BMDCs

In order to determine whether GM-CSF or FLT3L preferentially promoted DC differentiation towards CD103⁺ or E-Cadherin⁺ DCs *in vitro* mouse bone-marrow was cultured for 7days in the presence of GM-CSF (20ng/ml) or for 9 days in the presence of FLT3L (200ng/ml).

A) Representative Flow cytometry data. CD103 or E-cadherin expression was determined relative to isotype controls on live (7-AAD), CD11c⁺ cells.

B) Collated data from multiple experiments. Statistical significance was determined using a Mann-Whitney test (*p<0.05).

Each data point represents the data from a single independent experiment

4.2.2 CD103⁺ DC differentiation is enhanced by combined treatment with FLT3L and GM-CSF.

Emerging evidence that expression of one of the key features of CD103⁺DCs, the enzyme ALDH1A2, could be induced by sequential treatment of bone-marrow progenitors with FLT3L and then GM-CSF *in vitro* made us question whether perhaps combined treatment with both these factors might enhance the generation of CD103⁺ DCs in our cultures[341]. Consistent with that hypothesis, addition of GM-CSF to cultures after 6 days differentiation in the presence of FLT3L, caused a substantial and dramatic increase in the proportion of CD103⁺ DCs (~60%, **Figure 4.2**).

A characterisation of the surface marker phenotype of the CD103⁺DCs generated by sequential FLT3L and GM-CSF addition (**Figure 4.2B**) showed that they had begun to express CD11b and the macrophage marker F4/80 at low-levels very similar to those we had identified in the small intestinal lamina propria (Chapter 3, [24]). Furthermore, although pDCs also differentiate in response to FLT3L and GR1⁺ monocytes could be detected in cultures differentiated with GM-CSF, the CD103⁺DCs we generated did not express either B-220 or GR1 showing that they were neither pDCs nor monocytes. In addition, they uniformly expressed MHC-II at moderate levels, but not the non-conventional MHC-I molecule, CD1d. A proportion of CD103⁺ DCs characterised in lymphoid tissue expressed the DC subset marker CD8 α although the CD103⁺ DCs in the lamina propria did not ([60] Chapter 3). The CD103⁺ DCs generated *in vitro* remained CD8 α ⁻, consistent with reports that CD8 α is not inducible by FLT3L alone or by GM-CSF addition [391]. CD8 α can be induced by DC maturation [391, 437] and this might indicate that CD8 α is a molecule inducible by the tissue environment. Importantly, the CD103⁺

DCs generated by addition of GM-CSF remained clearly E-Cadherin⁻ consistent with the distinction of these DC phenotypes *in vivo* [39]. Furthermore, there was no increase in the proportion of E-Cadherin⁺ DCs after addition of GM-CSF to FLT3L cultures which, after 6 days, were already mostly of a DC and non-monocytic phenotype. This further supports the suggestion that E-Cadherin⁺ DCs do not derived from a FLT3L-induced lineage.

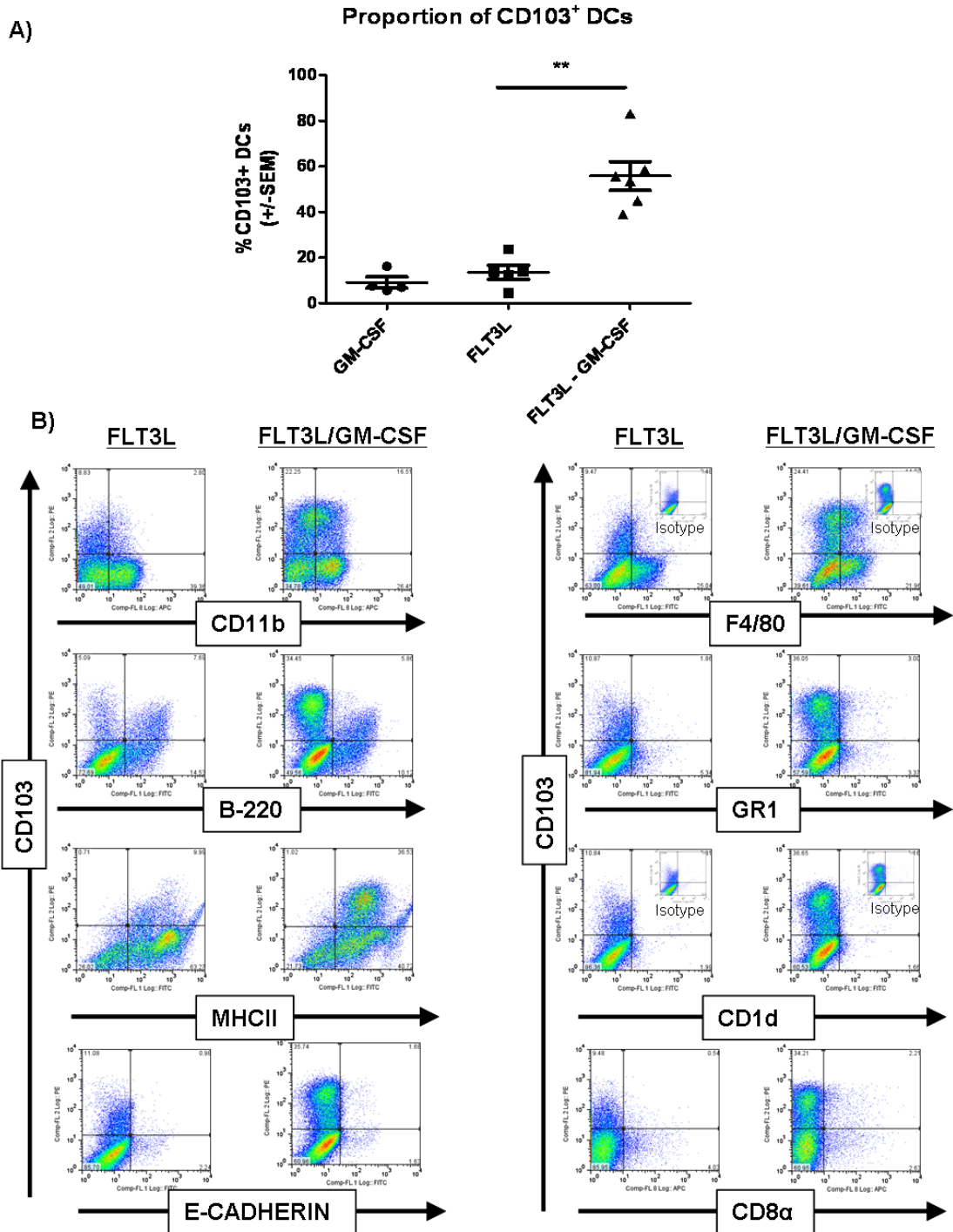


Figure 4.2 CD103⁺ DC differentiation is enhanced by culture with FLT3L and GM-CSF

A) Bone marrow was cultured for 8 days in the presence of GM-CSF (20ng/ml) or FLT3L (200ng/ml) or with FLT3L for 6days followed by addition of GM-CSF for 48hrs. The proportion of CD103⁺ amongst live (7-AAD⁻), CD11c⁺ cells was determined by flow cytometry.

Each data point represents an independent experiment.

Statistical significance was determined by Mann-Whitney test (**p<0.01)

B) The expression of various surface markers on *in vitro* differentiated CD103⁺ DCs determined by flow cytometry.

GR1 and B-220 expression representative of two independent experiments. Remaining markers representative of one experiment

4.2.3 Development of CD103⁺ DCs *in vitro* is not dependent on TGF- β

CD103 is a gene known to be regulated by TGF- β and it has been hypothesised that CD103 expression by DCs may be indicative of exposure to TGF- β *in vitro* [397, 398]. Therefore, we tested whether the generation of CD103⁺ DCs by culture with FLT3L and GM-CSF was an indirect result of TGF- β production. Using an anti-TGF- β neutralising antibody, which was able to inhibit the TGF- β induced FoxP3⁺ CD4⁺ T cell induction *in vitro* (**Figure 4.3B**), we found no effect of TGF- β neutralisation on the generation of CD103⁺ DCs in our system (**Figure 4.3A**).

4.2.4 Maturation of DCs *in vitro* reduces expression of CD103

In order to elicit full T cell responses DCs require maturation, which induces the production of cytokines and expression of co-stimulatory markers such as CD40, CD80 and CD86. Interestingly, CD103⁺ DCs generated *in vitro* had a slightly reduced basal level of CD40, CD80 and CD86 expression than CD103⁻ DCs. However, maturation of DCs with LPS up-regulated the expression of these markers to equivalent levels. In addition, we observed a consistent down-regulation in expression of CD103 itself after maturation with TLR stimuli but not CD40-ligation. This was true both in the mean-fluorescent intensity of CD103⁺ DCs (**Figure 4.4A**) and the proportion of CD103⁺ DCs in the cultures (**Figure 4.4B**).

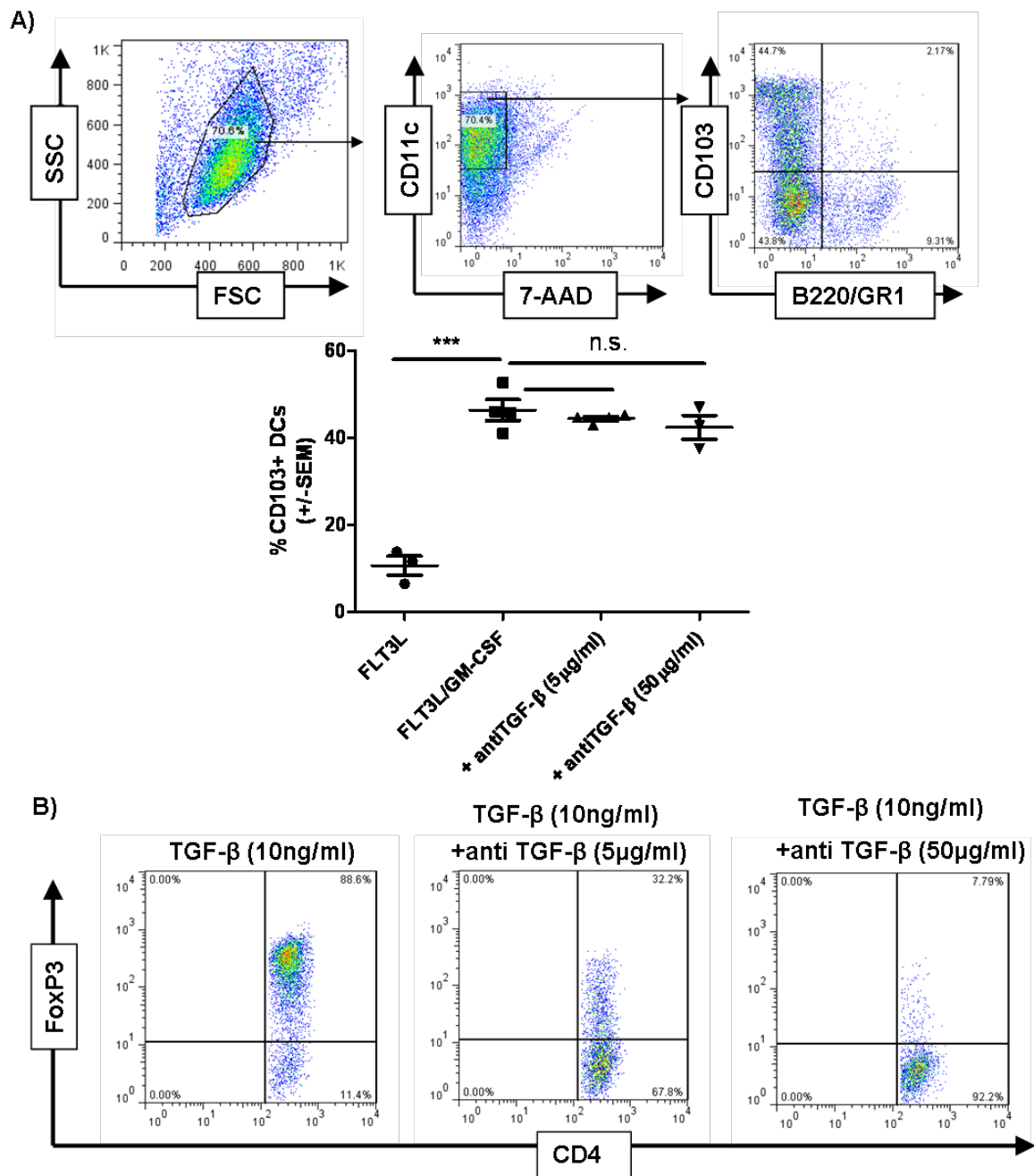


Figure 4.3 The differentiation of CD103⁺ DCs *in vitro* is not dependent on TGF- β

- A) Bone marrow was treated with FLT3L for 8days or with FLT3L for 6days followed by addition of GM-CSF for the final 48hours. Anti-TGF- β (5 μ g/ml or 50 μ g/ml) was added concurrent with GM-CSF and the proportion of CD103⁺ DCs amongst live (7-AAD⁻), CD11c⁺ non-pDCs/monocytes (B-220/GR1⁺ cells) in cultures was determined by flow cytometry. Statistical significance was determined by Matt-Whitney test (***p<0.001)
- B) As a positive control for the activity of anti-TGF- β antibody FoxP3⁺ Tregs were induced by anti-CD3/CD28 stimulation for 3 days in the presence of TGF- β (10ng/ml) and increasing concentrations of anti-TGF- β antibody. The neutralisation of FoxP3-induction was determined by intracellular staining and flow cytometry.

Data represents n=3 biological replicates from two independent experiments.

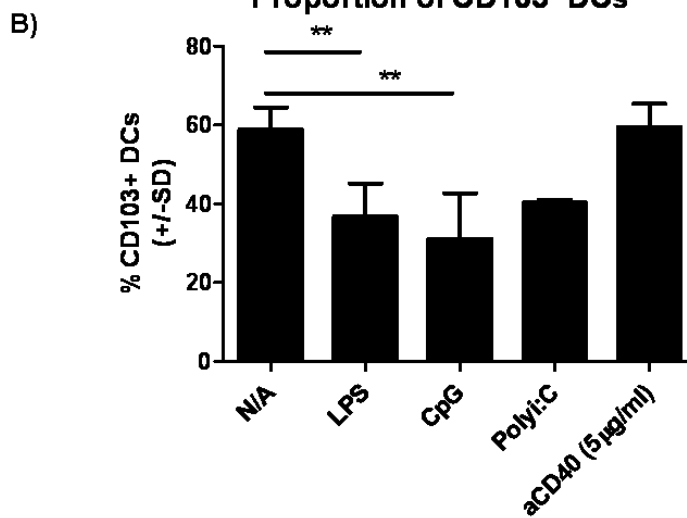
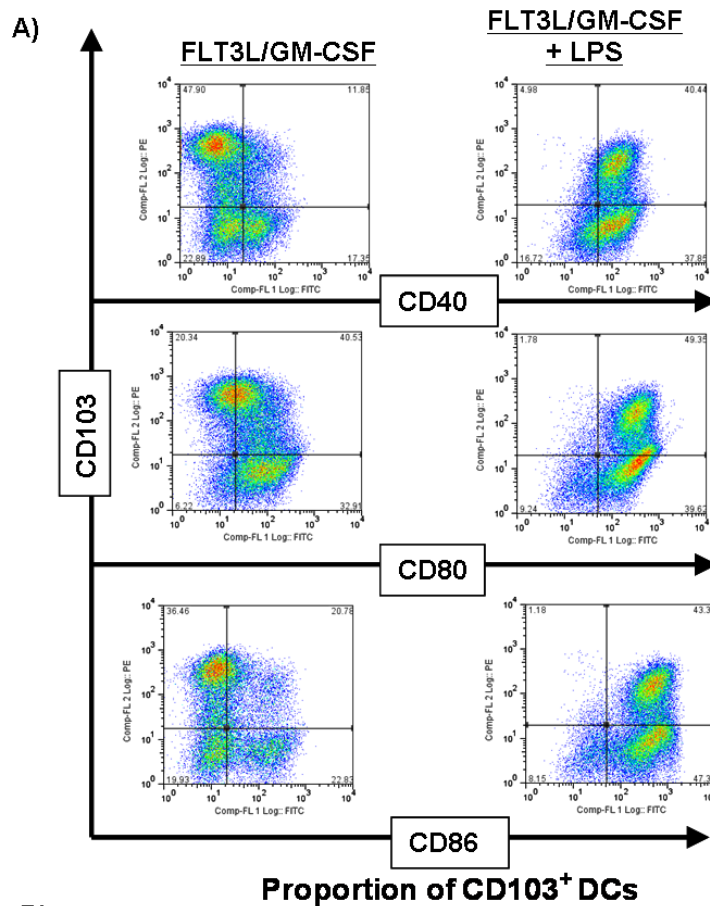


Figure 4.4 CD103⁺ DCs generated *in vitro* are not refractory to LPS-induced maturation, but expression of CD103 is reduced after maturation with TLR stimuli

A) CD103⁺ DCs differentiated by sequential addition of FLT3L and GM-CSF were treated for 24hr with LPS (1µg/ml) and the expression of co-stimulatory molecules (CD40, CD80 and CD86) assessed by flow cytometry. Data representative of one experiment

B) Collated data assessing the proportion of BMDCs expressing CD103 after sequential addition of FLT3L and GM-CSF (N/A) and further treatment with LPS (1µg/ml), CpG (10µM), Poly:I:C (100µg/ml) or anti-CD40 antibody (5µg/ml).

Data for LPS and CpG is pooled from n=3-4 biological replicates from two independent experiments. Data for Poly:I:C and antiCD40 represents n=2 from two independent experiments.

4.2.5 ALDH expression and activity by CD103⁺ DCs is controlled by TLR stimuli *in vitro*.

The aldehyde dehydrogenase (ALDH), RALDH2, encoded by the gene ALDH1A2, is a key enzyme catalysing the irreversible production of retinoic acid from retinal *in vivo* and is highly expressed in intestinal CD103⁺ DCs [157, 335, 338]. Preliminary experiments had indicated that the ALDH1A2 expression was elevated after the treatment of BMDCs or splenic DCs with LPS or CpG *in vitro* (**Figure 4.5A**). We therefore investigated how TLR stimuli might regulate ALDH activity on a single cell level using the Aldefluor assay (described in detail in chapter 3). Briefly, in the presence of active ALDH enzymes a mildly fluorescent substrate is converted to a highly fluorescent product. Therefore, Aldefluor positivity indicates elevated ALDH activity. A small proportion of DCs differentiated with FLT3L/GM-CSF treatment were positive for Aldefluor prior to maturation however the majority of these did not express CD103 (**Figure 4.5B**). The maturation of these DCs with LPS, CpG or Poly(I:C), but not anti-CD40 induced substantial increases in the proportion of Aldefluor⁺ DCs primarily amongst the CD103⁺ DC population (**Figure 4.5B**). This shows that TLR stimuli were sufficient to induce ALDH activity *in vitro*.

The inability of anti-CD40 treatment to induce ALDH activity could have been due to the lack of significant CD40 expression observed on *in vitro* differentiated DCs prior to maturation. Therefore, we investigated whether addition of anti-CD40 during maturation, when CD40 levels are elevated, had any synergistic effect on the acquisition of Aldefluor positivity. Surprisingly, however, anti-CD40 treatment in fact significantly inhibited the Aldefluor activity of the cells suggesting that this differential maturation may alter the DCs in a way that blocks the acquisition of a retinoic acid producing phenotype (**Figure 4.6**).

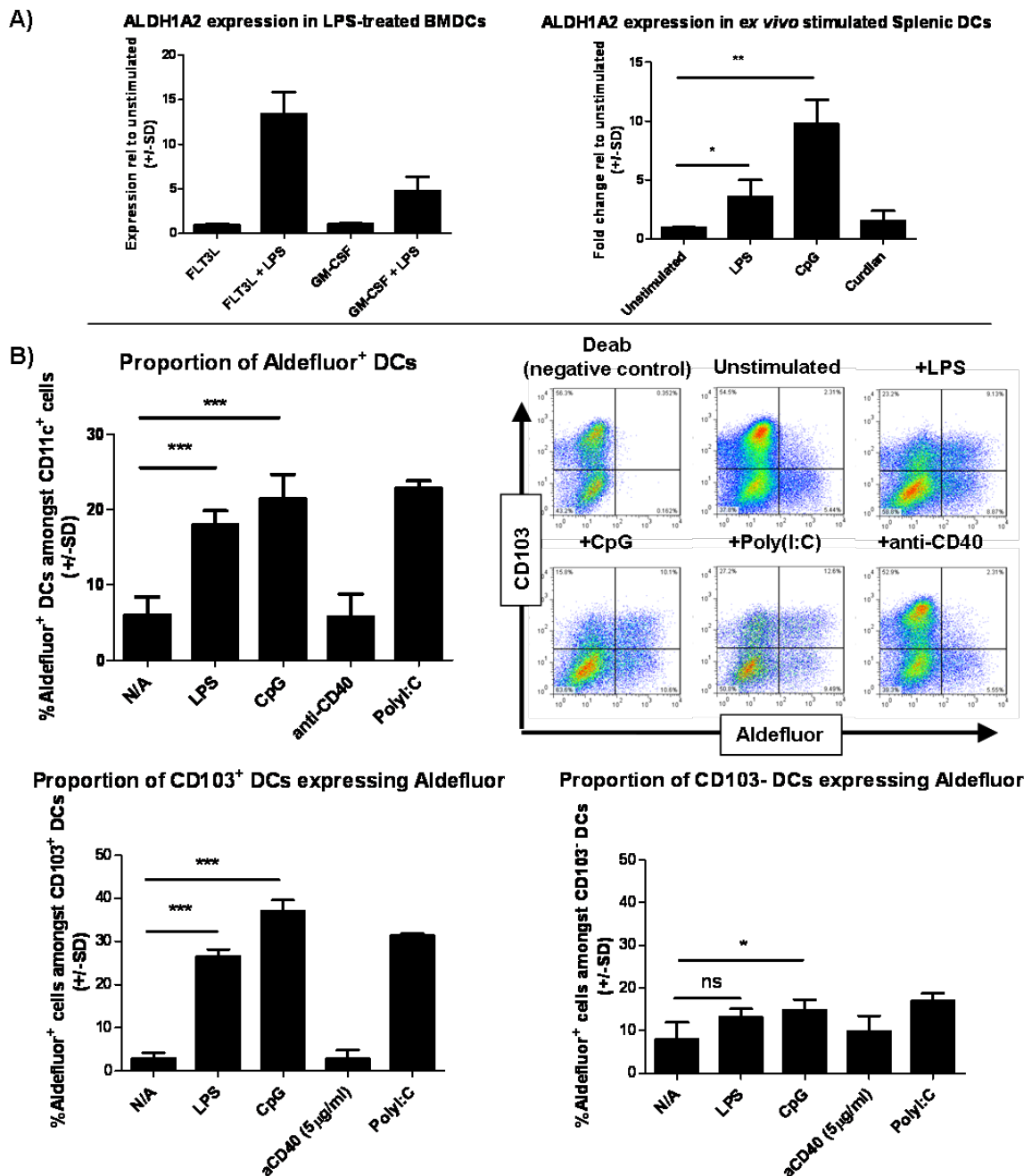


Figure 4.5 ALDH expression and activity are regulated by TLR-stimuli *in vitro*

A) In preliminary experiments, RNA was extracted from GM-CSF or FLT3L BMDCs after 8 days or BMDCs stimulated with LPS for the final 24 hrs of culture or from magnetically enriched splenic CD11c⁺ cells which had been cultured overnight or stimulated overnight with LPS (50 ng/ml), CpG (10 µM) or Curdlan (50 µg/ml). The expression of the enzyme ALDH1A2 was determined by Q-PCR. Data represents n=3 replicates from one experiment.

B) Using a fluorescent assay for ALDH-activity, aldefluor, the activity of RALDH2 was assessed by flow cytometry in CD103⁺ and CD103⁻ BMDCs generated by sequential culture with FLT3L and GM-CSF and treated for the last 24 hrs with LPS (1 µg/ml, n=3), CpG (100 µM, n=3), anti-Cd40 (5 µg/ml, n=3) or Poly(I:C) (100 µg/ml, n=2) or unstimulated (N/A) control conditions (n=4). Activity was measured relative to a negative control using an inhibitor of ALDH activity (Deab).

Data is pooled from three independent experiments (CpG, LPS and anti CD40) and two experiments (Poly(I:C)). Statistical significance determined using a Student's *t*-test.

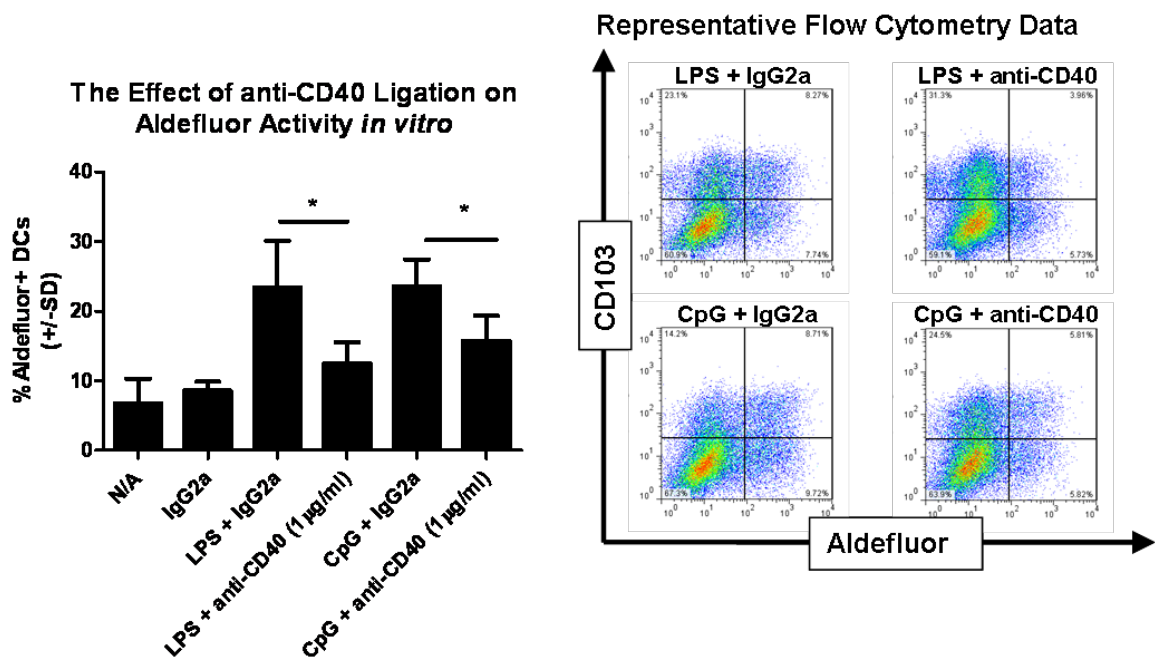


Figure 4.6: Aldefluor activity is inhibited by CD40-agonism

CD103⁺ DCs were differentiated with sequential addition of FLT3L and GM-CSF and treated with LPS (1 µg/ml, n=4) or CpG (10 µM, n=3) in the presence and absence of anti-CD40 (1 µg/ml, n=4) or isotype control (IgG2a, n=4). ALDH activity was determined by Aldefluor expression amongst live (7-AAD⁻) CD11c⁺ DCs.

Data represents the mean +/- SD from three independent experiments. Statistical significance was determined using a Student's *t*-test (**p*<0.05)

4.2.6 Retinoic acid substantially induces ALDH activity *in vitro*.

Although TLR stimuli *in vitro* were able to induce ALDH activity, our subsequent studies *in vivo* indicated that this was unlikely to have a significant role in controlling ALDH activity during homeostasis (see chapter 3). The partitioning of ALDH activity between the small and the large intestine *in vivo* suggested that perhaps a dietary factor was more likely to be promoting ALDH activity during homeostasis. Given that retinoic acid itself can be derived from the diet via vitamin A we investigated whether retinoic acid itself would be sufficient to induce ALDH activity *in vitro*. Indeed, retinoic acid potently increased the proportion of Aldefluor⁺ DCs *in vitro* (~80%) to a level above that induced by maturation with TLR-stimuli alone (Figure 4.7). This suggested that retinoic acid itself could control the generation of ALDH⁺ DCs in the intestine.

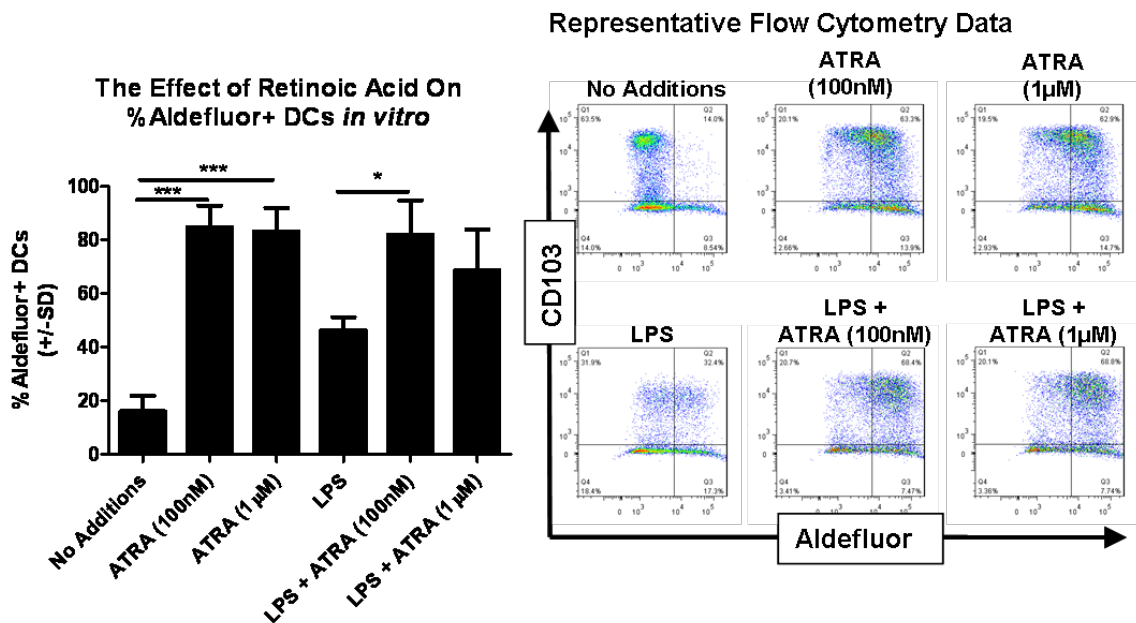


Figure 4.7 All trans retinoic acid (ATRA) potentially induces ALDH-activity *in vitro*. CD103⁺ BMDCs were differentiated *in vitro* by sequential addition of FLT3L and GM-CSF and treated for 24 hours with retinoic acid (ATRA) in the presence or absence of LPS. Statistical significance was determined using a Mann Witney Test (*p<0.05, **p<0.01, ***p<0.001). Data represents the mean +/- SD of n=3 from three experiments

4.2.7 CX3CR1⁺ CD103⁻ DCs are also generated in cultures with FLT3L and GM-CSF

Our on-going studies of dendritic cells in the lamina propria had identified distinct populations of CX3CR1⁺CD103⁻ DCs (chapter 3). It was therefore of interest to determine whether these were also generated in this *in vitro* system and whether they remained distinct from CD103⁺ DCs. The culture of CX3CR1^{GFP/+} bone-marrow showed clearly the presence of CX3CR1⁺ DCs, which formed a distinct CD103^{-/lo} BMDC population, mirroring the partitioning observed amongst these subsets isolated from the lamina propria (**Figure 4.8**).

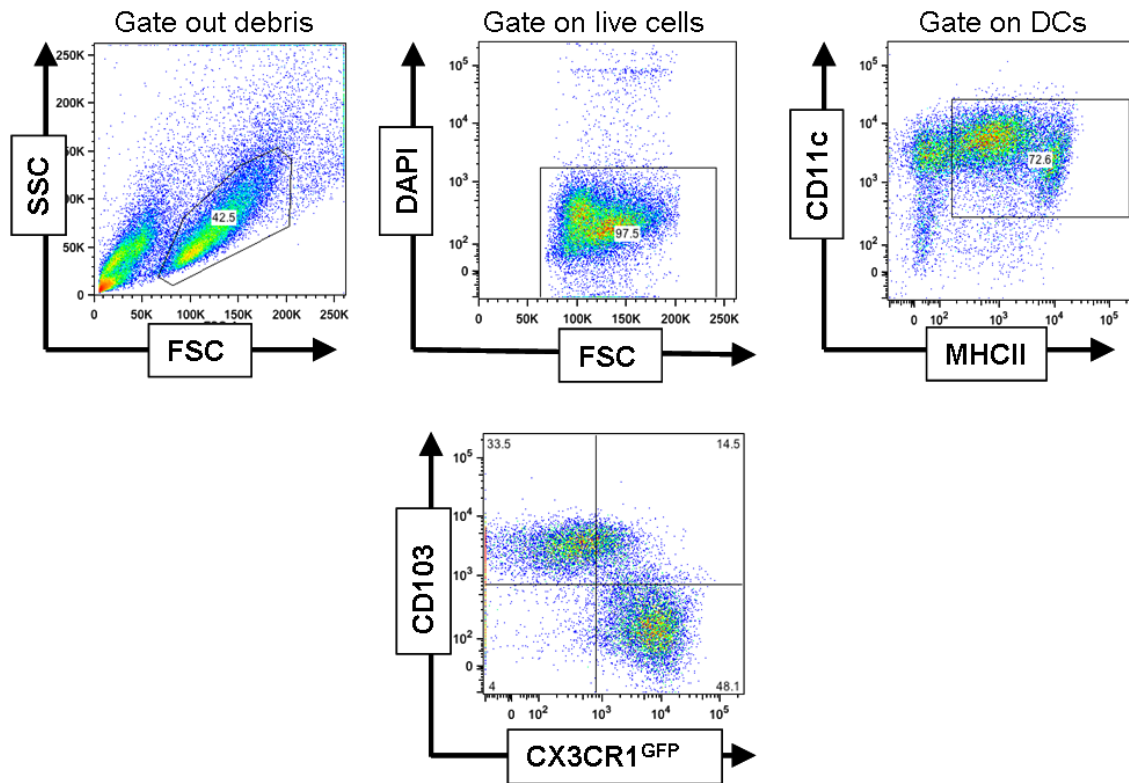


Figure 4.8 Distinction of CD103⁺ and CX3CR1⁺ DCs is apparent in *in vitro* cultures

CD103⁺ BMDCs were differentiated from CX3CR1^{GFP} bone marrow by sequential addition of FLT3L and GM-CSF. After 9 days BMDCs were analysed for expression of CD103 and CX3CR1 by flow cytometry.

Data representative of n=3 biological replicates from one experiment

4.2.8 Induction of FoxP3⁺ CD4⁺ T cells by CD103⁺ and CD103⁻ BMDCs

One important feature of CD103⁺DCs isolated from the small intestine or the MLN is the preferential ability to induce FoxP3⁺ Treg cells particularly in the presence of exogenous TGF- β [157, 158]. Given the enhanced development of CD103⁺ DCs induced by FLT3L and GM-CSF *in vitro* we analysed the ability of these cells or their CD103⁻ counterparts to induce FoxP3 expression in co-culture with MACS-enriched CD4⁺CD45Rb⁺ T cells (**Figure 4.9**). Interestingly, in the absence of any additional maturation CD103⁺ DCs differentiated *in vitro* had a substantially enhanced ability to induce FoxP3 expression than CD103⁻ DCs isolated from the same cultures. This feature suggests an “innately tolerogenic” nature capacity for

this CD103⁺ DC subset determined simply by FLT3L and GM-CSF differentiation factors without an essential requirement for exposure to signals specific to the intestinal environment. In fact, the maturation of BMDCs overnight with bacterial LPS completely ablated this ability to promote FoxP3⁺ CD4⁺ T cell differentiation despite the enhanced ALDH activity we had previously detected in these cultures using the Aldefluor assay. This therefore suggests that the tolerogenic phenotype of CD103⁺ DCs is not irreversible and supports recent indications that the ability of retinoic acid does not necessarily promote FoxP3⁺Treg cell differentiation in all conditions[438]. By contrast treatment of BMDCs overnight with retinoic acid (ATRA) marginally enhanced the ability of CD103⁺ and CD103⁻ DCs to promote FoxP3⁺CD4⁺ T cell differentiation suggesting that exposure to retinoic acid which is present in the intestinal environment at high levels may act to enhance the tolerogenic properties of intestinal DCs.

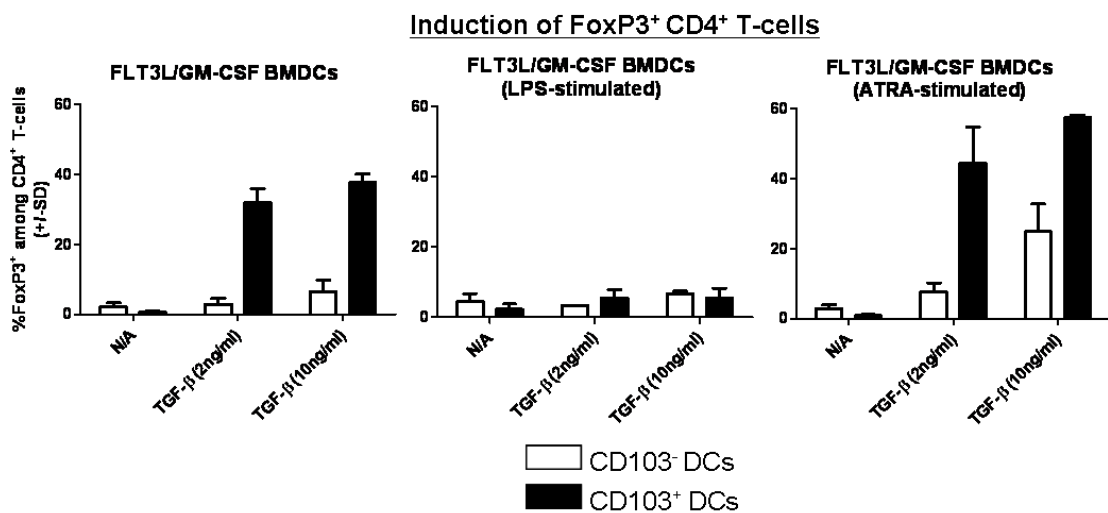


Figure 4.9: *In vitro* generated CD103⁺ DCs have a preferential ability to induce FoxP3 which is ablated by LPS-stimulation

BMDCs were differentiated by sequential addition of FLT3L and GM-CSF and either untreated (control, n=3) or treated with LPS (1 μg/ml, n=3) or ATRA (100nM, n=2) for the final 24hrs of culture. CD103⁺ and CD103⁻ DCs were then isolated to high purity by FACS and 3x10⁴ BMDCs were co-cultured with 2 x 10⁵ magnetically-purified CD4⁺CD45Rb⁺ T-cells and anti-CD3 (0.5 μg/ml). Cultures were supplemented with either no additions (N/A) or specified concentrations of TGF-β. After 3 days, CD4⁺ TCR-β⁺ cells were identified and the expression of FoxP3 determined by intracellular staining and flow cytometry.

Data representative of the mean +/- SD from n=3 biological replicates from one experiment.

4.3 Discussion

We describe here the development of an *in vitro* culture system which gives rise to CD103⁺ DCs in culture that have a natural predisposition to promote FoxP3⁺ CD4⁺ T cell differentiation and can be induced towards a high ALDH-activity phenotype, using retinoic acid or TLR-ligation. Therefore, these *in vitro* generated CD103⁺ DCs appear similar to CD103⁺ DCs isolated from the small intestine and MLN (see chapter 3, [157, 158]).

Substantial, extremely elegant work over the past few years has described the ontogeny pathway for conventional lymphoid tissue DC development and it is worth taking a moment to put the development of CD103⁺ DCs described here into this context. It is evident that the direct precursor of lymphoid tissue DCs, the CD11c⁺MHCII⁻ pre-DC, circulates from the bone-marrow and seeds both lymphoid and non-lymphoid tissue giving rise to conventional DC subsets [23, 27, 435]. Although the development of this precursor is not strictly dependent on FLT3-signalling *in vivo*, the numbers of pre-DCs and even earlier DC progenitors, such as the over-lapping macrophage-DC-progenitors (MDPs) and common DC-precursors (CDPs), are expanded in FLT3L-treated mice [28]. Similarly, proliferating precursors can be identified within FLT3L-driven BMDC cultures and these sequentially differentiate into cDCs *in vitro* [27]. The careful characterisation of FLT3L^{-/-} and FLT3L^{-/-}GM-CSF^{-/-} mice shows clearly that the differentiation of pre-DCs to cDCs and their maintenance within the spleen is governed almost exclusively by FLT3L during homeostasis with little requirement for GM-CSF [26, 28, 430, 434]. Within the spleen only few DCs express CD103 (~10-25%) [60] and consistent with this FLT3L-driven development program we show that only few CD103⁺ DCs arise within BMDC cultures supplemented with FLT3L alone (~10%).

Furthermore, CD103⁺DCs within the spleen and also within FLT3L BMDC cultures are CD11b⁻ and both express comparatively lower levels of CD103 than the cells with this phenotype isolated from the small intestine or MLN [60].

Rather than depending on FLT3L alone, the differentiation of CD103⁺ DCs *in vitro* is reliant on the addition of GM-CSF to FLT3L BMDC cultures after 6 days and culturing for a further 24-48hrs. Importantly, these growth factor requirements described for CD103⁺ DC differentiation *in vitro* mirror precisely the requirements recently described for small intestinal CD103⁺ DC differentiation *in vivo*. This is clear from the substantial and specific reductions in small intestinal CD103⁺ DCs evident in both FLT3^{-/-} and GM-CSFR^{-/-} mice [24]. It is also significant that adoptive transfer of pre-DCs could give rise to CD103⁺ DCs in the intestines, whereas the transfer of monocytes could not [24, 25]. Interestingly, colonic CD103⁺ DCs are equally reliant on FLT3L, but not reliant on GM-CSF for their development [25]. This is consistent with colonic CD103⁺ having distinct functional properties, including a lack of ALDH activity (see chapter 3).

It should be noted that the *in vivo* requirements for FLT3L and GM-CSF and our *in vitro* requirements for FLT3L and GM-CSF for the differentiation of CD103⁺ DCs are at odds to other culture systems in which CD103⁺ DC development has recently been described. In particular the development of CD103⁺ DCs was demonstrated within GM-CSF-driven BMDC cultures and this was suggested to be dependent on the production of TGF- β and retinoic acid by intestinal epithelial cell lines grown in trans-wells [397]. By contrast, we found no requirement for TGF- β in CD103⁺ DC differentiation in our culture system. Furthermore, the use of GM-CSF alone as a growth factor in the published system [436], which is generally thought to produce monocyte-derived cells, makes interpretation of the data

difficult. Particularly given that the *in vivo* studies clearly stress a requirement for FLT3L and show the contribution of monocyte-derived cells to CD103⁺ DCs in the steady-state to be negligible [24, 25, 436]. Indeed, our cultures with GM-CSF alone showed the substantial development of E-Cadherin⁺ DCs which we have previously shown to be monocyte-derived and are more inflammatory both *in vitro* [436] and *in vivo* [39]. Therefore, collectively the work discussed above emphasises that purely GM-CSF driven, monocyte-derived lineages are not appropriate to model steady-state cDC responses *in vitro* and this would include the modelling of intestinal CD103⁺ DCs.

The time-point for GM-CSF addition (day 6) was driven by a desire to skew the differentiation of FLT3L-driven DCs and pre-DCs rather than acting directly on monocytes and monocyte-derived cells. Consistent with this, most non-sustained cells die off between day 1-4 of FLT3L cultures [27] and there a few GR1⁺ cells, indicative of monocytes, remaining in culture after day 5 [391] whereas by day 8 most cells are already of a DC phenotype (CD11c⁺MHCII⁺). However, it would be interesting to determine at precisely which point of the lineage GM-CSF acts to induce this specialised CD103⁺ DC phenotype. Achieving this might indicate points at which the pathway could be manipulated without altering the rest of the DC network. Analysis of the kinetics of BrdU-labelling have indicated that CD103⁺ DCs are unlikely to arise from CD103⁻DCs *in vivo* [439] and therefore it seems likely that GM-CSF must act at the level of a DC precursor perhaps upon entering the small intestinal environment. Interestingly, analysis of FLT3 and GM-CSFR expression on myeloid cell precursors showed minimal expression in haematopoietic progenitors or MDPs [26] (consistent with the redundancy of both FLT3L and GM-CSF for precursor development *in vivo* [28]). However expression

of both FLT3 and to a lesser extent GM-CSFR was substantial on CDPs and the derivatives [26], suggesting that GM-CSF is likely to act at this stage of differentiation. Consistent with this suggestion CX3CR1⁺c-kit⁻ cells which are likely to represent CDPs isolated from the bone-marrow gave rise to substantial proportions of CD103⁺ DCs when cultured with GM-CSF for 5 days [440]. Unfortunately, this population was not characterised further to assess its similarity to *in vivo* CD103⁺ DCs as the authors focused on cultures derived from MDPs which are likely to contain substantial numbers of contaminating monocyte-derived cells.

We have used this *in vitro* system to investigate factors which might be additionally involved in inducing ALDH activity, which is a key feature of intestinal CD103⁺ DCs thought to be essential for their ability to produce the co-factor retinoic acid [157, 158, 335, 338]. We found that both TLR-stimuli and retinoic acid itself were able to induce ALDH activity suggesting that the microbiota or the metabolism of dietary vitamin A *in vivo* may both contribute to the full development of CD103⁺ DC phenotype. The relative contributions of both these possibilities were discussed in detail in chapter 3. However, given that in the absence of additional stimuli CD103⁺ DCs differentiated with FLT3L and GM-CSF were naturally able to enhance FoxP3⁺ CD4⁺ T cell responses it is possible that these host-derived signals alone are sufficient to endow CD103⁺ DCs with an *in vivo* phenotype and that direct exposure to additional environmental signals (so-called “DC conditioning”) is considerably less important than has been hypothesised. Consistent with this it has been noted that the constitutive maturation and migration of DCs in the GALT is independent of bacterially-derived signals [64, 360, 361]. Instead the regulation of GM-CSF production *in vivo* may be critical to

maintain CD103⁺ DC differentiation. Recent studies have indicated that GM-CSF in the MLN and small intestine can be produced at low levels by epithelial cells and at a higher level by steady-state macrophages [341]. Interestingly, GM-CSF production by macrophages was ablated in vitamin A-deficient mice and GM-CSF production by epithelial cells is responsive to the presence of pathogenic bacteria [341, 441]. Therefore, it seems likely that CD103⁺ DCs are regulated primarily by controlling the GM-CSF driven differentiation of this subset perhaps in response to environmental factors, rather than being conditioned directly by environmental signals themselves.

The link between GM-CSF and the induction of CD103⁺ DCs apparently with a more tolerogenic phenotype raises the question about the therapeutic potential of targeting this axis *in vivo*. Hypothetically this could be achieved either directly by administering GM-CSF or indirectly perhaps by supplementation with vitamin-A or by exposure to probiotic bacteria. It is clear that GM-CSF administration can have beneficial effects on inflammatory diseases via the enhancement of tolerogenic DCs and the subsequent induction of regulatory T cell responses [442-446] and GM-CSF therapy has been trailed in Crohn's disease patients [447]. It would be interesting to see if GM-CSF administration expands intestinal CD103⁺ DCs and conferred enhanced protection in murine colitis models via preferential Treg cell induction. Conversely, as CD103⁺ DCs are hypothesised to be important for oral tolerance, analysing the induction of FoxP3⁺ iTreg cells and the development of tolerance towards oral antigen in GM-CSF^{-/-} or GM-CSFR^{-/-} mice would be a worthwhile experiment. During inflammation or infection, GM-CSF drives the generation of pro-inflammatory monocyte-derived DCs [36, 39], therefore its role in the intestine, like TGF- β and retinoic acid, is likely to be context dependent.

Retinoic acid is known to act directly on CD4⁺ T cells to enhance FoxP3 expression and inhibit the development of Th17 cells [156, 332, 328, 448, 329, 331]. Interestingly, however we found that maturation with LPS ablated the preferential ability of *in vitro* derived CD103⁺DCs to promote FoxP3 expression despite enhancing the ALDH activity of BMDCs. Furthermore, we found that anti-CD40 ligation appeared to blunt the induction of ALDH activity suggesting that neither of these features of CD103⁺ DCs are permanent in nature, but rather that they can be reversed depending on the context. This supports emerging evidence that retinoic acid functions in a pleiotropic, concentration dependent manner and is not always deemed to be a tolerogenic factor. Indeed it is apparent that retinoic acid can also promote Th17 responses, particularly following stimulation of small intestinal DCs with flagellin [310, 311], and vitamin-A deficiency has long been associated with the impairment of immune responses against infectious agents [333]. Similarly, CD103⁺ DCs are no-longer able to preferentially support FoxP3⁺ Treg cell differentiation when isolated from inflamed conditions induced by T cell transfer [439], where interestingly the CD40L-CD40 interaction is a major inflammatory pathway [449, 450]. Finally elegant studies using vitamin A-deficient mice and T cell specific RAR α ^{-/-} mice have shown that retinoic acid signalling is critical for proper T cell activation and this may underlie the different T cell responses observed in varying levels of retinoic acid-deficiency or sufficiency [399, 400, 438]. Although, we did not observe any differences in T cell proliferation in our cultures (determined by CFSE-dilution) nor any up-regulation of IL-17 responses following LPS-maturation of BMDCs, further experiments using a greater range of antigen or anti-CD3 concentrations, a greater range of TGF- β concentrations and blockade of retinoic acid or cytokine signalling, such as by IL-6, may indicate better how the CD103⁺ DC phenotype can be altered by bacterial,

cytokine or co-stimulatory signals to promote more pro-inflammatory T cell responses.

Finally, the technical difficulties isolating and studying human intestinal DCs are even more exaggerated than for the mouse making the development of appropriate *in vitro* systems for human DC analysis even more critical. Currently, the isolation of peripheral blood monocytes and the generation of monocyte-derived DCs by culture with GM-CSF/IL-4 represent the predominant mainstream protocol for studying human DCs [29], but for the reasons outlined above this is unlikely to be suitable for modelling CD103⁺ DCs in the steady-state. In translation to the human FLT3L administration has shown to expand the number of peripheral blood in healthy volunteers suggesting a similar pathway is active in the human as has been described in the mouse [451]. Furthermore, expansion of DC precursors and to a lesser extent full differentiation to mature DCs, has been described after culture of CD34⁺ progenitor cells with FLT3L in combination with other growth factors such as thrombopoietin (TPO) and stem-cell factor (SCF) [452, 453]. However, it seems likely that complete differentiation into CD1a⁺ DCs is dependent on the treatment of FLT3L-generated precursors with GM-CSF, perhaps in a manner not dissimilar to our murine culture system [452]. However neither expression of CD103 in human DCs derived *in vitro* nor their similarity to small intestinal CD103⁺ DCs (whose phenotype is conserved between mouse and human [396]) has been assessed. We are therefore attempting to translate the culture system described in this chapter to human studies using both cord blood and embryonic stem cells as progenitors. Indeed, stem cell technology has allowed for the generation of M-CSF driven macrophages [454] and GM-CSF driven, monocyte-derived DCs [455] *in vitro*. The ability to use stem cells as

progenitors would provide a more regular supply of DCs which are also more amenable to functional studies than primary cells. In addition, such technology raises the possibility of generating DCs from IBD patients and also from individuals harbouring IBD-relevant polymorphisms. Indeed, induced-pluripotent stem cells (iPS cells), which could be generated from IBD-patients pending ethical approval, have been used to differentiate macrophages *in vitro* (S. Cowley, W. James, personal communication). Such an advance would enable substantially more complete study of human DC phenotypes in IBD patients and allow functional characterisation the various immune pathways which genetic studies have implicated in causing IBD pathogenesis.

Chapter 5

The influence of the microbiota on regulatory T cell responses in the intestine

5.1 Introduction

As described in the general introduction both FoxP3⁺ Treg cells and FoxP3⁻ CD45Rb^{lo} CD4⁺ T cells can have regulatory functions in the intestine [124, 245, 248, 274]. Furthermore, FoxP3⁺ Treg cells can be separated into nTreg cells, derived in the thymus, and iTreg cells, derived in the periphery [252]. The gut-associated lymphoid tissue is thought to be a preferential site for iTreg cell induction and this is hypothesised to offer necessary supplementation of the Treg cell pool at this location to meet the elevated antigenic potential [152, 246, 314]. A key mechanism by which both FoxP3⁺ Treg cells and FoxP3⁻ CD45Rb^{lo} CD4⁺ T cells mediate immune-regulation is via the production of IL-10 [245, 274, 280, 288]. The absolute dependency of FoxP3⁻ CD45Rb^{lo} CD4⁺ T cell regulation on IL-10 production suggests these cells may be similar to Tr1 cells. Tr1 cells can be generated *in vitro* by repeated antigen stimulation in the presence of IL-10 [128], by vitaminD3 and dexamethasone [144] or with IL-10-conditioned DCs [149, 299]. However, canonical CD4⁺ Th cells, Th1, Th2 and Th17, can all be induced to produce IL-10 depending on co-stimulation (e.g. via Notch [456] or ICOS [457]) or cytokine stimulation (e.g. with IL-2 [301, 302]). Therefore, given the potential heterogeneity of this population, here we have termed intestinal FoxP3⁻IL-10⁺ CD4⁺ T cells “Tr1-like”.

Recent evidence has shown that the certain effector functions of CD4⁺ T cells in the lamina propria are dependent on the microbiota. Germ-free mice, which lack a

microbiota, or mice treated with broad spectrum antibiotics [163, 185] have substantial reductions in the proportion of intestinal Th17 cells [201, 226-228]. Furthermore, Th17 cell differentiation has been linked specifically to the presence of Segmented Filamentous Bacteria (SFB) [201]. However there is also evidence that SFB acts more widely to promote effector T cell differentiation notably including substantial IFN- γ production [226]. Although there is no clear evidence that the proportion FoxP3⁺ Treg cells are altered in germ-free mice [250, 376, 458] there is strong evidence that the microbiota plays a role in regulating their function [245, 274]. For example, oral infection with the bacteria *Helicobacter hepaticus* (*H. hepaticus*) increases the regulatory activity of both Treg cells (CD25⁺CD45Rb^{lo}) and Tr1-like cells (CD25⁺CD45Rb^{lo}) *in vivo* [274]. However, precise mechanisms determining the impact of the microbiota on CD4⁺ T cells remain unclear.

DCs are thought to be the key communicators relating the intestinal lamina propria environment to CD4⁺ cell responses. However, little is known about the effect of microbiota depletion on DC phenotype in the lamina propria. DC migration from the lamina propria appears intact in germ-free rats [64] and there was little alteration in the proportions of different DC subsets, their maturation status or their *in vitro* antigen presenting function when analysed from the spleen or MLN of germ-free mice compared to controls [360, 361]. Emerging evidence has however suggested that microbial colonisation of neonates may induce the recruitment of DCs to the intestinal lamina propria [362].

We set out to determine the effect of modulating the microbiota (with broad-spectrum antibiotic-treatment or by infection with *H. hepaticus*) on the proportion of

DC subsets and the induction and IL-10-producing capacity of FoxP3⁺ Treg cells and Tr1-like cells in the intestine.

5.2 Results

5.2.1 The effect of antibiotic treatment on lamina propria myeloid cell subsets

Establishing completely germ-free mice requires a dedicated facility with gnotobiotic isolators access to which is extremely limited within the UK. Furthermore, we were primarily interested in uncovering a direct role for the microbiota in modulating DC function and were concerned that a failure of the immune system to develop the correct architecture in germ-free animals [178, 179, 205] may make it difficult to interpret the results. Therefore, we allowed the mice to be colonised normally after birth and then employed an established protocol of broad-spectrum antibiotic treatment to deplete the microbiota in the intestine [185]. This protocol has been shown to cause a 100 fold reduction in the amount of 16S rRNA in the stool and results in a make up of 16S rRNA sequences almost identical to those derived from autoclaved food, suggesting the depletion of an endogenous microbiota [394]. This protocol has also been sufficient to uncover marked alterations in intestinal Th17 cells following antibiotic treatment [227].

Consistent with previous reports we observed substantial enlargement of the caecum in mice treated with antibiotics similar to that observed in germ-free mice [459] caused by a reduced ability to digest complex carbohydrate (data not shown). However, an analysis of CD103⁺ DCs, CX3CR1^{int} cells and CX3CR1^{hi} cells in the colon and small intestine of antibiotic treated mice indicated that there was no significant alteration in the proportion or total number of these cell subsets

relative to controls (**Figure 5.1**). In the caecum, which had undergone gross anatomical changes, there was a substantial increase in proportion and total number of CX3CR1^{hi} cells and a slight increase in CD103⁺ DCs, suggesting that these cell populations might be sensitive to these changes.

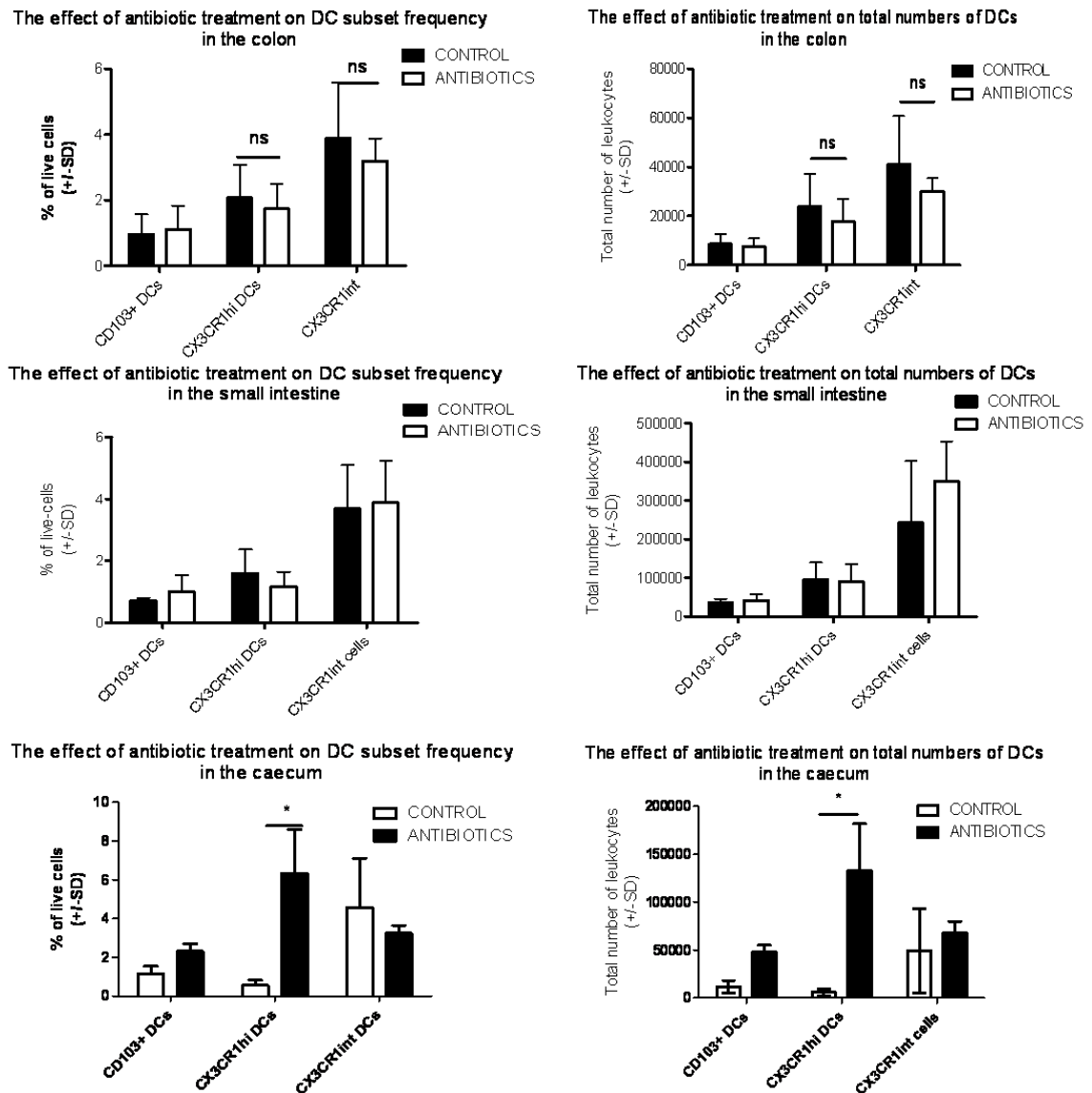


Figure 5.1 Antibiotic treatment does not cause significant alteration in DC subset frequency or number in the lamina propria

CX3CR1^{GFP/+} mice were treated with normal drinking water (control) or water supplemented with broad spectrum antibiotics (antibiotics) for four weeks. Cell suspensions were generated by collagenase digest of the tissues indicated and DC subsets identified by co-staining with CD103, MHCII and CD11c as previously described (see figure 4.8). Except where indicated no statistical significance was determined using a student's *t*-test. Significant values shown were determined using a Mann Whitney test (**p*<0.05). Data represent pooled data from three independent experiments for colon (*n*=12), two independent experiments for small intestine (*n*=9) and one experiment for caecum (*n*=3).

5.2.2 The effect of *Helicobacter hepaticus* infection on intestinal myeloid cell subsets

Under homeostatic conditions there are several mechanisms in place to ensure that the immune system remains largely ignorant of the microbiota. These include a substantial mucous layer, antimicrobial peptides and IgA-secretion [172]. This barrier prevents bacteria from colonising the crypts or adhering to the intestinal epithelium. *H. hepaticus* is an intestinal bacterium capable of penetrating the mucous layer to colonise the crypts of the colon and caecum and also the bile duct of the liver [460]. It therefore resides in closer proximity to the immune cells, including DCs, than the majority of intestinal bacteria species. Furthermore, even in wild-type mice, systemic IgG antibodies can be detected against *H. hepaticus* indicating that the immune system is not ignorant of its presence [278]. We investigated whether infection with *H. hepaticus* resulted in any active alterations in either the proportion or total number of DC subsets. Infection of mice with *Helicobacter hepaticus* failed to cause any significant alteration in the proportion or total number of CD103⁺ DCs, CX3CR1^{int} cells or CX3CR1^{hi} cells at either day 3, day 7 or day 14 after infection (**Figure 5.2**).

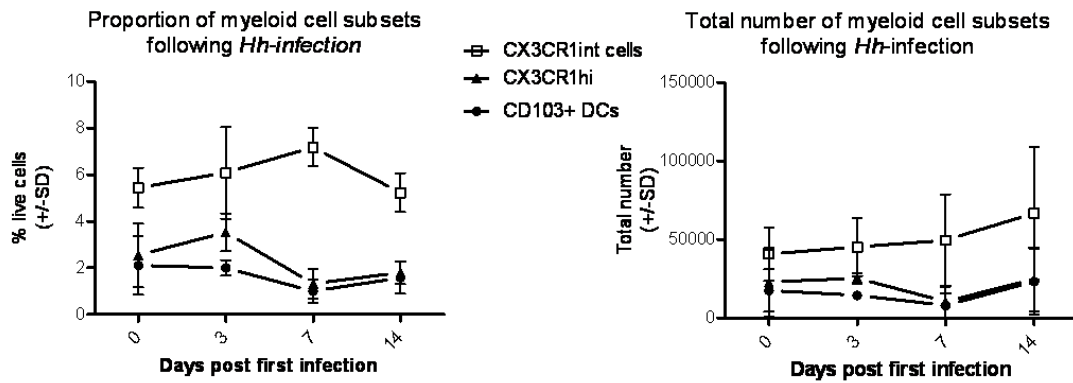


Figure 5.2 *Helicobacter hepaticus* infection does not induce substantial accumulation of CD103⁺ DCs, CX3CR1^{int} or CX3CR1^{hi} cells

CX3CR1^{GFP/+} mice were infected with 1×10^8 *H. hepaticus* bacteria on 3 consecutive days starting at day 0. The proportion and total number of CD103⁺ DCs, CX3CR1^{int} cells and CX3CR1^{hi} cells prior to infection on day 0 (n=6) or at day 3 (n=4), day 7 (n=4) or day 14 (n=4) post first infection was determined from live colonic LPLs by flow cytometry.

Data represent the mean proportion or total number of myeloid cell subsets from n=5 biological replicates from one experiment. A second experiment gave similar results

5.2.3 The effect of antibiotic treatment on the *in vivo* response to orally administered antigen

Although we could detect little role for the microbiota in modulating the gross numbers or the ALDH activity of intestinal DCs (see chapter 3) we decided to more widely investigate their antigen presenting capability *in vivo* by modelling the response to oral antigen. OVA₃₂₃₋₃₃₉-specific naive CD4⁺ T cells from DO11.10SCID mice (KJ1.26⁺ cells) were adoptively transferred to recipient mice which had either a SPF microbiota or had been treated with antibiotics. For two consecutive days after cell transfer all mice received ovalbumin by oral gavage. Ovalbumin is processed *in vivo* resulting in presentation of the OVA₃₂₃₋₃₃₉ epitope and the activation of transferred cells as measured by the appearance of substantial proportions of Ki67⁺ KJ1.26⁺ cells. In addition, the induction of FoxP3⁺ KJ1.26⁺ cells 5 days after ovalbumin administration is a feature of the process of oral tolerance [157, 158].

In antibiotic treated mice there was a significant reduction in the proportion of Ki67⁺ KJ1.26⁺ T cells that were induced by ovalbumin in the spleen and the MLN, but only minimal reductions in the small intestine and the Peyer's Patches indicating a modest defect in the proliferative response of the transferred cells (**Figure 5.3A**). In addition following ovalbumin administration, there was a clear induction of FoxP3⁺ KJ1.26⁺ T cells, which was in fact elevated in the MLN and small intestine of antibiotic-treated animals (**Figure 5.3B**). Therefore, it is apparent that, although the microbiota has a minor role in stimulating the proliferative response of CD4⁺ T cells to oral antigen, it is likely dispensable and may even be slightly inhibitory to the induction of FoxP3⁺ Treg cells.

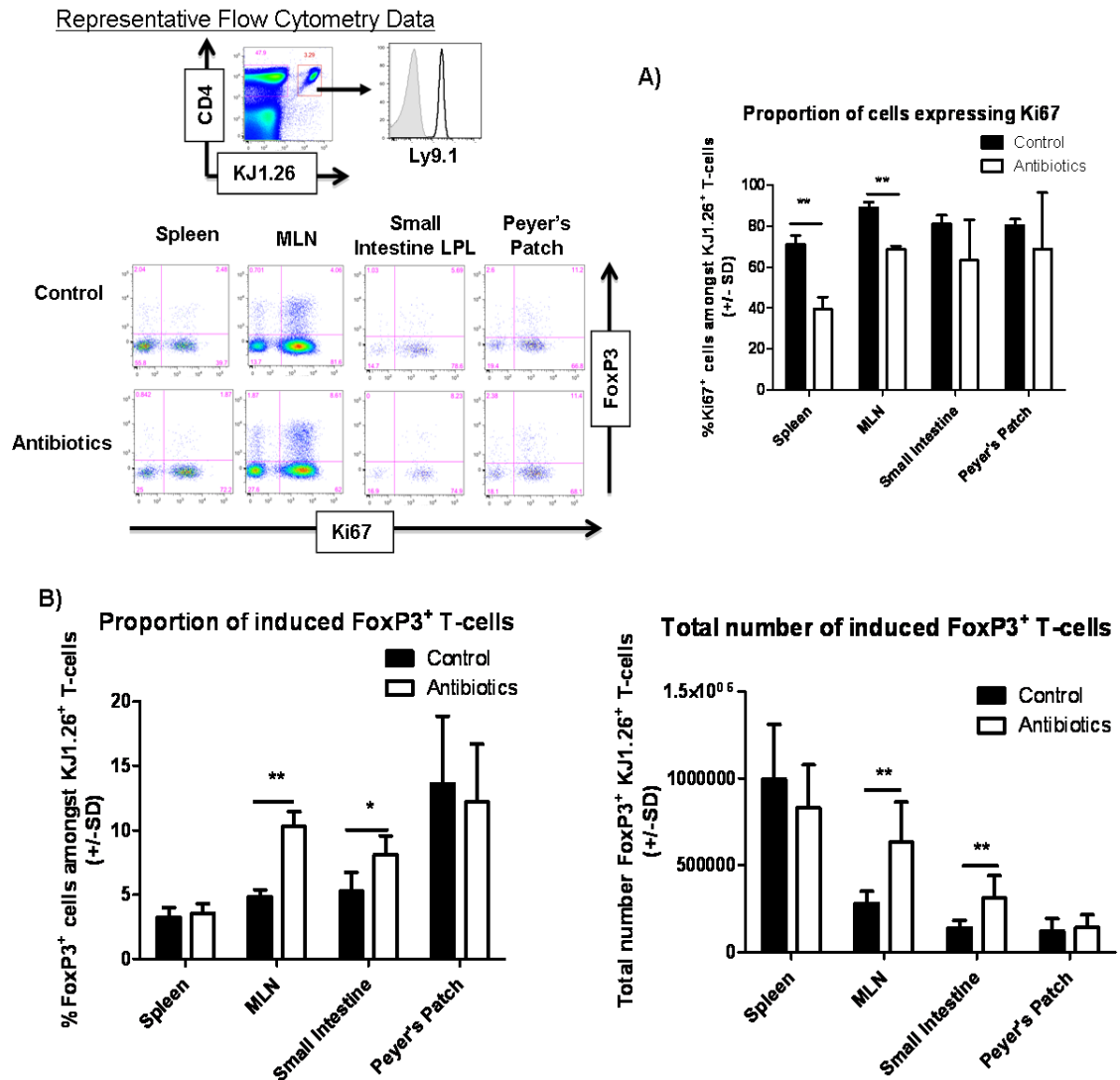


Figure 5.3 Antibiotic treatment has minor effects on the proliferative response to oral antigen and enhances FoxP3⁺ Treg cell induction

Ly9.2 mice received antibiotics (n=5) or water (control, n=5) by daily gavage for 14 days. After 14 days 5x10⁶ CD4⁺ T-cells (all of which are FoxP3⁺) from the lymph nodes of DO11.10SCID mice were adoptively transferred into Ly9.2 animals. For two days after transfer mice were provided with 50mg ovalbumin by oral gavage and antibiotic treatment in the relevant mice was continued for a further 5 days. The proportion of transferred KJ1.26⁺Ly9.1⁺CD4⁺ T-cells expressing (A) Ki67 and (B) FoxP3 was then determined by tissue digest and flow cytometry using relevant antibodies. Significance was determined using a Mann Witney test (*p<0.05, **p<0.01).

Data represent the mean of n=5 biological replicates from one experiment.

5.2.4 The effect of antibiotic treatment on IL-10 production by Treg and Tr1-like cells in the intestine

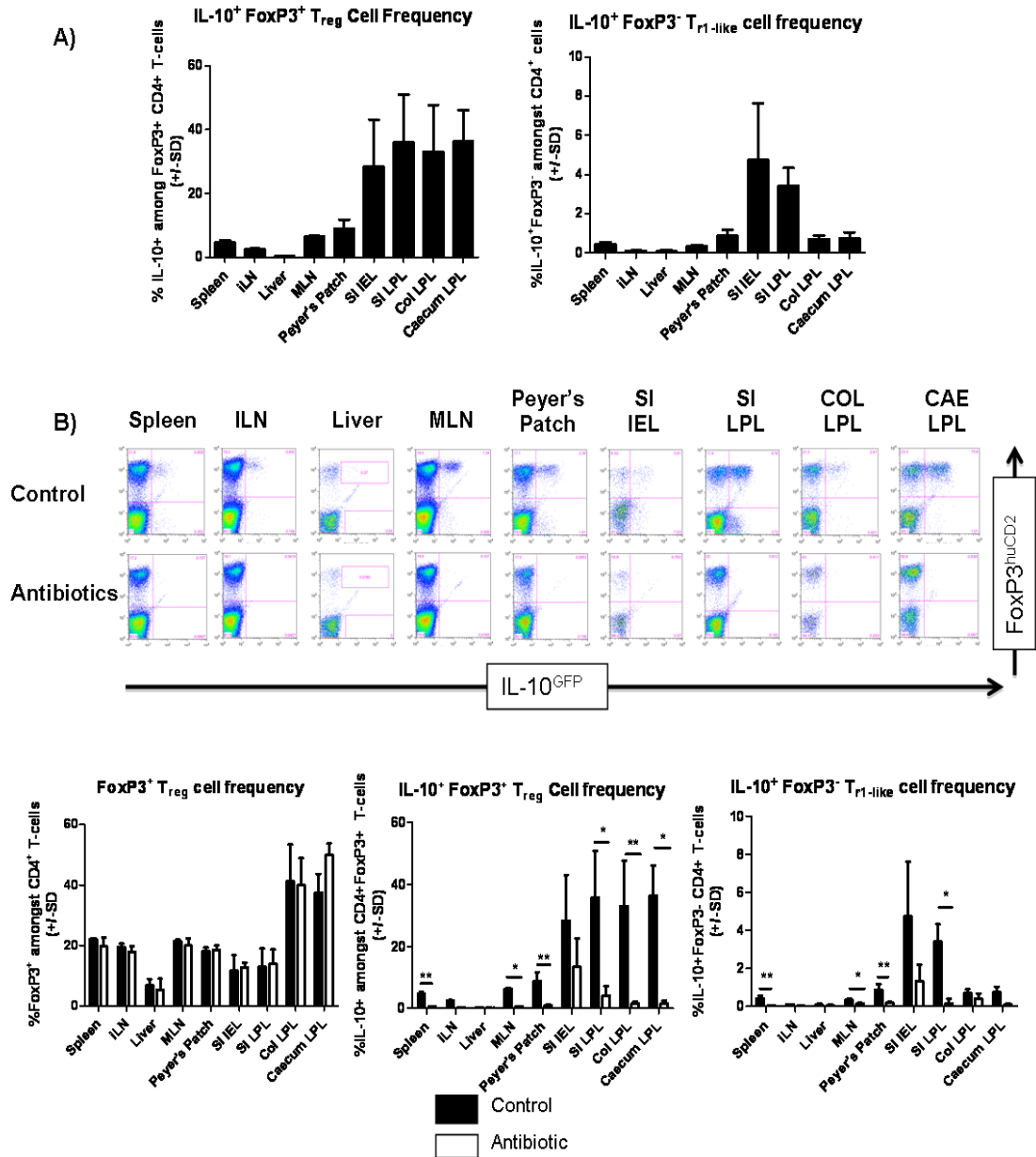
Emerging reports have shown that germ-free or antibiotic treated mice have reduced cytokine production from effector CD4⁺ T cells [201, 226, 227]. We

therefore decided to analyse further the IL-10 production by regulatory T cells following antibiotic treatment. Using a FoxP3^{huCD2}IL-10^{GFP/+} dual reporter mouse we were able to analyse IL-10 production from both FoxP3⁺ and FoxP3⁻ T cells without any requirement for further manipulation (M. Barnes, A. Johnson, J. Bollrath, manuscript in preparation). This mouse was generated by crossing the IL-10^{GFP/GFP} (*TIGER*) strain [405] to a strain expressing a signalling-deficient human CD2 (huCD2) construct under control of the FoxP3 promoter [461] (breeding of this mouse strain was supervised by M. Barnes).

An analysis of IL-10^{GFP} expression amongst FoxP3⁺ Treg cells indicated a strong enrichment of IL-10 production in the small intestine, colon and caecum relative to tissues peripheral to the intestine such as the spleen, inguinal lymph node (iLN) and liver (**Figure 5.4A**). The proportion of FoxP3⁻ Tr1-like cells expressing IL-10 (henceforth referred to as Tr1-like cells) was considerably lower than for FoxP3⁺ cells consistent with early reports [263]. However, there remained an enrichment of Tr1-like cells in the small intestine IEL and LPL relative to other *in vivo* locations (**Figure 5.4A**). It is also worth noting that the relative expression of IL-10^{GFP} in CD4⁺ T cells as measured by the Δ MFI greatly dwarfed that observed in myeloid CX3CR1^{hi} cells (see chapter 3).

Consistent with the lack of dependency of iTreg cell induction on the microbiota, there was no reduction in the overall proportions of FoxP3⁺ Treg cells in any of the tissues analysed when FoxP3^{huCD2}IL-10^{GFP/+} mice were treated with antibiotics. However, antibiotic treatment of FoxP3^{huCD2}IL-10^{GFP/+} dual reporter mice did cause a dramatic ablation of both FoxP3⁺IL-10⁺ and FoxP3⁻IL-10⁺ CD4⁺ T cells not only in the small intestinal, colonic and caecal lamina propria but also in the MLN, iLN and even the spleen (**Figure 5.4B**). Therefore, these results show that IL-10

production by both FoxP3⁺ Treg cells and FoxP3⁻ Tr1-like cells is dependent upon the intestinal microbiota.



5.2.5 Co-stimulatory molecule expression on IL-10⁺ CD4⁺ T cells

One mechanism by which cytokine production by T cells can be controlled involves signalling through co-stimulatory molecules [279]. In order to investigate whether intestinal IL-10 production might be regulated in this manner we analysed the expression of various co-stimulatory molecules on FoxP3⁺ Treg cells and FoxP3⁻ Tr1-like cells in the MLN and the small intestinal and colonic lamina propria. Generally, we found that FoxP3⁺ Treg cells expressed higher levels of ICOS, PD-1 and CTLA-4 than conventional CD4⁺ T cells (Tconv). Interestingly, amongst FoxP3⁺ Treg cells, we found that FoxP3⁺ IL-10⁺ Treg cells expressed higher levels of ICOS and CTLA-4 than FoxP3⁺ IL-10⁻ Treg cells, particularly in the MLN, suggesting that these may represent a more activated FoxP3⁺ Treg cell phenotype. By comparison, PD-1, was expressed equally among FoxP3⁺IL-10⁺ and FoxP3⁺IL-10⁻ Treg cells. Further definition with other surface markers such as CD62L, CD25, and Helios, an intracellular protein that is thought to be preferentially associated with nTreg cells rather than iTreg cells [462], may further indicate whether IL-10 expression is associated with recently activated FoxP3⁺ Treg cells. The Tr1-like cells of the small intestine did not show particularly elevated expression of ICOS, PD-1 or CTLA-4 relative to conventional CD4⁺ T cells (**Figure 5.5**).

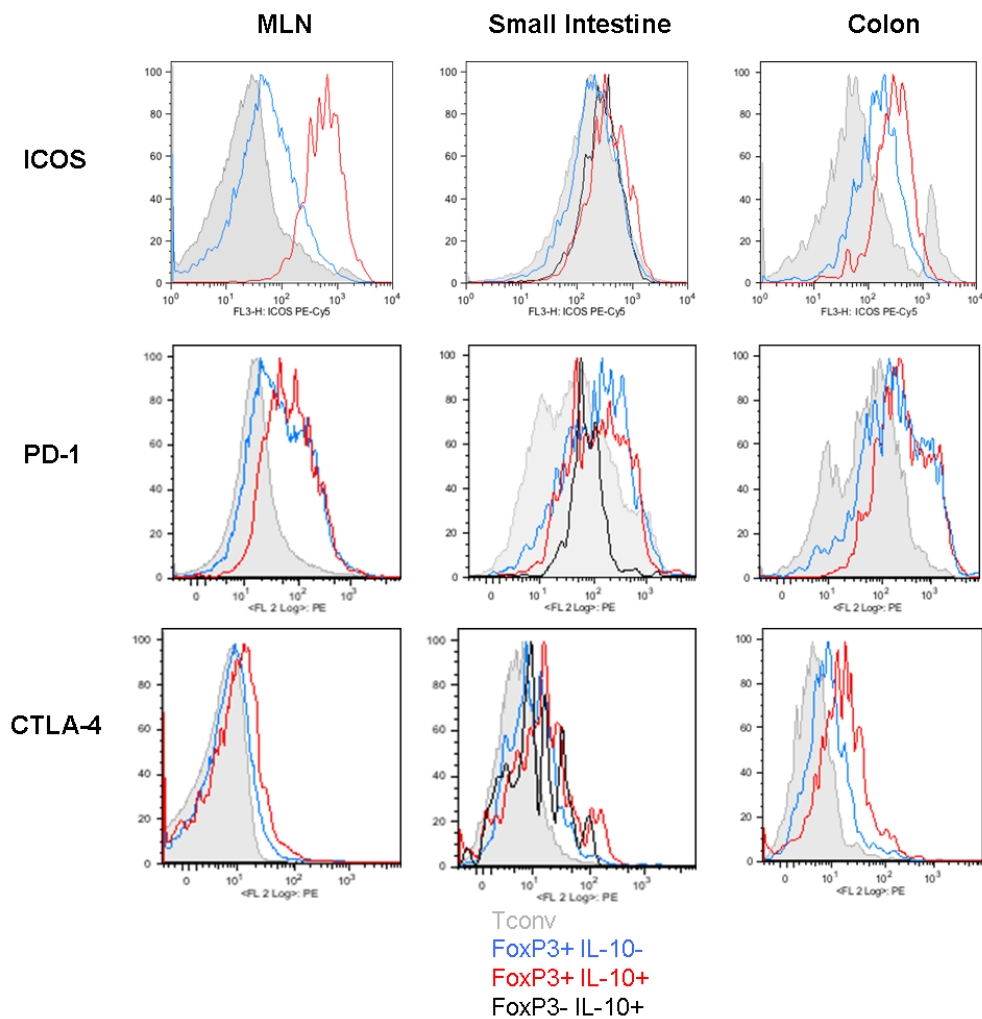


Figure 5.5 The expression of co-stimulatory molecules by FoxP3⁺, IL-10⁺ and IL-10⁻ conventional CD4⁺ T-cells

Single cell suspensions were generated from the mLN, Small Intestine LPL and Colon LPL of FoxP3^{huCD2}IL-10^{GFP/+} mice. Populations of FoxP3⁺IL-10⁻ conventional T-cells (Tconv, shaded area), FoxP3⁺ IL-10⁻ T_{reg} cells (blue line), FoxP3⁺IL-10⁺ T_{Reg} cells (Red line) and FoxP3⁻ IL-10⁺ T_{r1-like} cells (black line, small intestine only) were identified using fluorescent antibodies against CD4 and huCD2 respectively. Relative expression of ICOS, PD-1 and CTLA-4 was determined on different populations by flow cytometry.

Data representative of n=3 biological replicates from one experiment. A subsequent experiment by J. Bollrath gave similar results.

The ligands for these co-stimulatory molecules, ICOS-L, PDL-1, CD86 and CD80, were detectable on the intestinal myeloid cells described in chapter 3. We therefore investigated whether there was a loss of expression of these ligands resulting from antibiotic treatment which thus might explain the ablation of IL-10

producing CD4⁺ T cells. However, treatment with antibiotics did not alter the expression of ICOS-L, PDL-1, CD86 or CD80 on CD103⁺ DCs, CX3CR1^{int} cells or CX3CR1^{hi} cells suggesting that these interactions are unlikely to be the major regulators of IL-10 expression in the intestine (**Figure 5.6**).

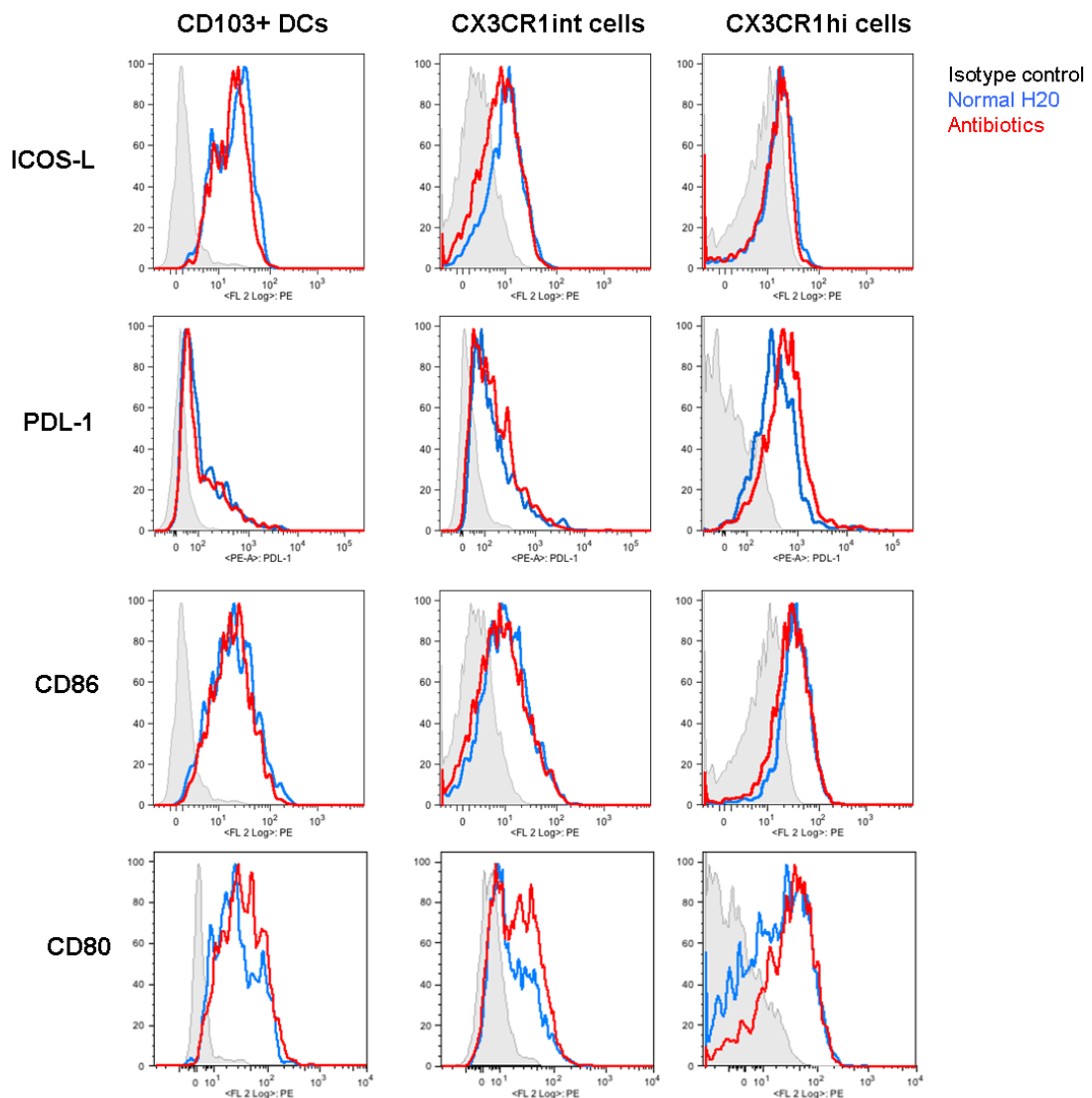


Figure 5.6: The expression of co-stimulatory molecules by colonic DC subsets are not altered by antibiotic treatment

Single cell suspensions were generated from the colon LPL of CX3CR1^{GFP/+} either treated for four weeks with antibiotics in drinking water (red line) or maintained on control water (blue line). DC subsets were identified as previously described with co-staining with CD103, MHCII and CD11c and the relative expression of specified molecules was determined relative to FMO control (shaded area).

Data representative of n=3 biological replicates from one experiment.

5.2.6 *Helicobacter hepaticus* enhances IL-10 production by CD4⁺ T cells in the lamina propria

The ability to tolerate colonisation with *H. hepaticus* in the colon and the caecum is dependent on IL-10 production [278] and CD25⁺ and CD25⁻ CD45Rb^{lo} CD4⁺ T cells have an enhanced regulatory activity when isolated from *H. hepaticus* infected mice [274] (M. Barnes, M. Asquith, K. Maloy, F. Powrie, Unpublished data). We therefore hypothesised that colonisation with *H. hepaticus* might promote IL-10 production by intestinal CD4⁺ T cells as part of a mechanism for tolerance. In support of this hypothesis we found that the proportion of FoxP3⁺ IL-10⁺ Treg cells and FoxP3⁻ IL-10⁺ Tr1-like cells in the colonic and caecal lamina propria was increased two weeks after infection with *H. hepaticus* relative to uninfected controls (**Figure 5.7A**). Interestingly, the enhanced IL-10 production was restricted to the lamina propria and was not observed in the MLN suggesting a localised effect. The increased IL-10 expression in the colon and caecum is unlikely to be the result of an influx of Treg cells that one might speculate could be “induced” in response to *H. hepaticus* as there were only minor increases in the proportion of FoxP3⁺ CD4⁺ T cells at this time-point (**Figure 5.7B**). Interestingly, feeding of heat-killed *H. hepaticus* to mice failed to enhance intestinal IL-10 production suggesting that constitutive molecular patterns deriving from bacterial components alone were insufficient, but rather an active process initiated by live bacteria is required (**Figure 5.7C**).

Therefore we conclude that intestinal IL-10 production by both FoxP3⁺ Treg cells and FoxP3⁻ Tr1-like cells is highly responsive to the composition of the microbiota, being ablated by antibiotic treatment and enhanced by *H. hepaticus* infection.

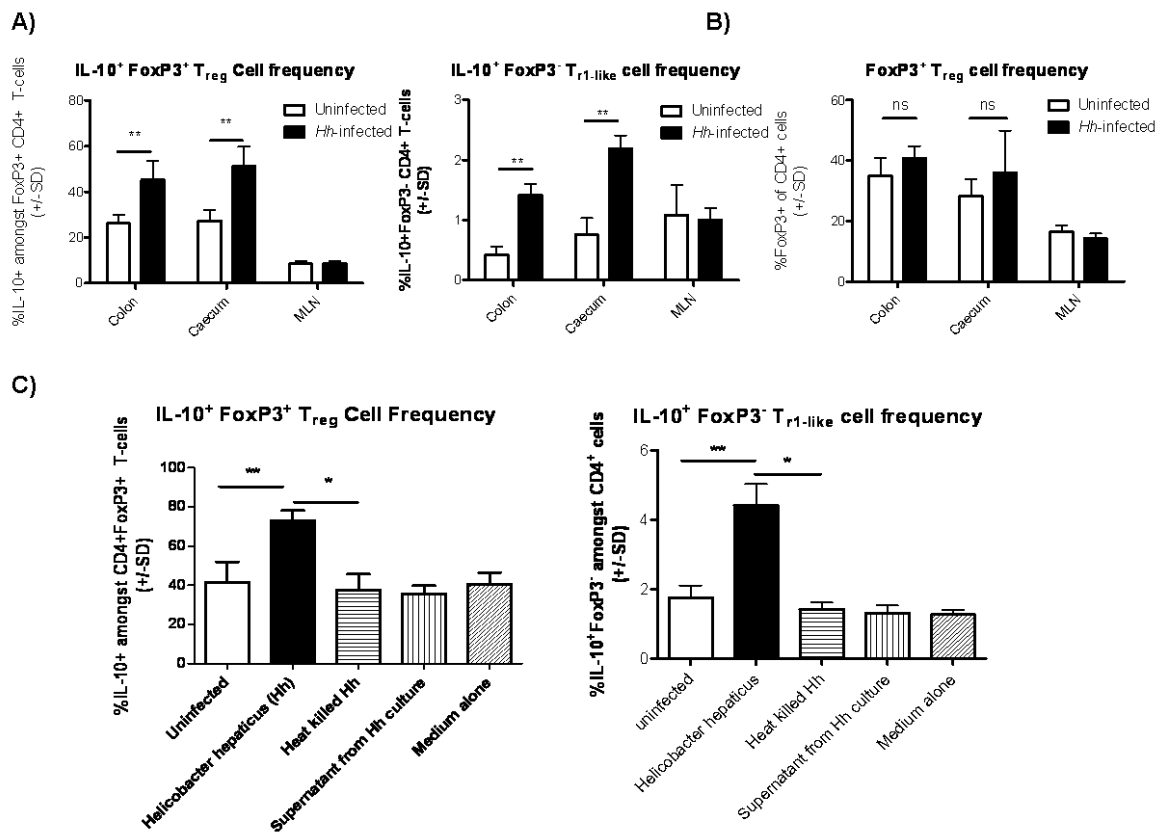


Figure 5.7 *Helicobacter hepaticus* enhances IL-10 production by intestinal CD4⁺ T-cells

A-B) FoxP3^{huCD2}IL-10^{GFP/+} mice were infected with 1×10^8 *H. hepaticus* bacteria by gavage on 3 consecutive days. After 14 days single cell suspensions were prepared from the colonic and caecal LPL and the mLN and the proportion of IL-10⁺ T_{reg} or T_{r1-like} were determined by co-staining with fluorescent antibodies against huCD2, CD4 and TCR- β .

Data represent the mean \pm SD of $n=4-8$ from two independent experiments. Further experiments by M. Barnes and J. Bollrath yielded similar results.

C) FoxP3^{huCD2}IL-10^{GFP/+} mice were infected with 1×10^8 *H. hepaticus* bacteria on 3 consecutive days. Alternatively, FoxP3^{huCD2}IL-10^{GFP/+} were treated with Heat killed *Helicobacter hepaticus*, supernatant from *Helicobacter hepaticus* anaerobic culture or culture media alone by daily gavage (repeated gavage by J. Bollrath). Proportions of IL-10⁺ T_{reg} or T_{r1-like} cells in pooled colon/caecal LPLs was determined by flow cytometry.

Data represent the mean \pm SD of $n=3-5$ biological replicates from one experiment.

5.2.7 IL-10 production by intestinal CD4⁺ T cells is independent of MyD88.

The ability of sentinel cells, such as DCs, to sense bacteria and induce effector function in CD4⁺ T cells, often involves the sensing of molecular patterns via pathogen recognition receptors such as TLRs. All known TLRs, except TLR3 and some responses of TLR4 require the adaptor protein MyD88 to signal [84]. The inflammatory response which is initiated by *H. hepaticus* in Rag^{-/-} mice or in IL-10-

deficient settings, fails to occur in MyD88-deficient settings, indicating that effects downstream of MyD88 drive *H. hepaticus* induced inflammation [198]. We therefore hypothesised that IL-10 production by CD4⁺ T cells, which is responsive to both *H. hepaticus* and the endogenous intestinal microbiota, would be dependent upon MyD88-signalling. To investigate this hypothesis we analysed the proportion of IL-10⁺ CD4⁺ T cells in the lamina propria of MyD88^{-/-}IL-10^{GFP/GFP} mice in the presence and absence of *H. hepaticus*. Surprisingly, we found no difference in the proportion of IL-10⁺ CD4⁺ T cells in the colon or caecum of MyD88-deficient mice compared to controls, either in uninfected animals or in mice infected with *H. hepaticus* for 14 days (**Figure 5.8**). This suggests no requirement for MyD88 signalling in the control of IL-10 production by intestinal CD4⁺ T cells by the microbiota or *H. hepaticus*.

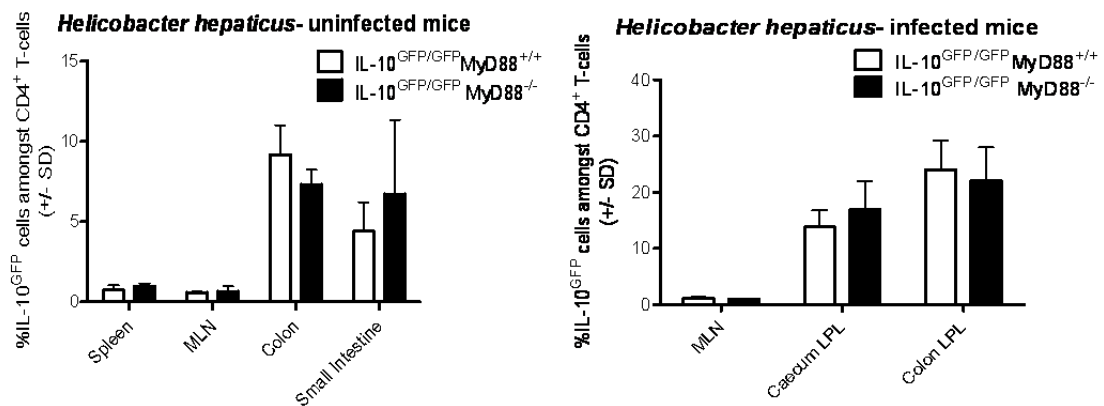


Figure 5.8: MyD88 is dispensable for IL-10 production by intestinal CD4⁺ T-cells

Single cell suspensions were generated from the specified organs of either uninfected mice or mice infected for 14 days with *H. hepaticus*. The proportion of CD4⁺ TCR-β⁺ cells expressing IL-10 was determined by flow cytometry. (Experiment conducted by M. Barnes and J. Bollrath, data analysed by A. Johnson).

Data represent the mean of n=3-5 biological replicates from one experiment. A second experiment on uninfected mice gave similar results.

5.2.8 IL-10 production by intestinal CD4⁺ T cells is independent of pDCs

IL-10 producing CD4⁺ T cells and FoxP3⁺ CD4⁺ T cells can both be induced by pDCs *in vitro* [325, 463]. In addition, pDCs influence the migration of cDC from the small intestinal lamina propria [326], play a role in tolerance of haptenated proteins [464] and the proportion of pDCs in lymphoid tissue appears to be responsive to changes in the microbiota [465]. We therefore hypothesised that IL-10 production by intestinal CD4⁺ T cells in response to the commensal flora or *H. hepaticus* infection would be dependent on pDCs. In order to test this hypothesis we used a pDC-depleting antibody (120G8) purified from the supernatant of a hybridoma cell line [388] to ablate pDCs in uninfected or *H. hepaticus*-infected FoxP3^{huCD2}IL-10^{GFP/+} mice. A preliminary experiment indicated depletion of pDCs in all tissues analysed just 24 hours after injection of antibody (data not shown). This data and evidence from literature protocols [395, 466] resulted in us settling on injections three times per week at 48 hour intervals in order to maintain pDC-depletion for a two week period. For the experiment involving subsequent infection with *H. hepaticus* we provided two injections of antibody prior to infection and then continued injections for a two week period. Despite apparent depletion of pDCs, there was no alteration in the proportion of FoxP3⁺ IL-10⁺ Treg cells or FoxP3⁻IL-10⁺ Tr1-like cells either before or after *H. hepaticus* infection (**Figure 5.9A-C**). This indicated that pDCs are likely dispensable for the production of IL-10 by intestinal CD4⁺ T cells.

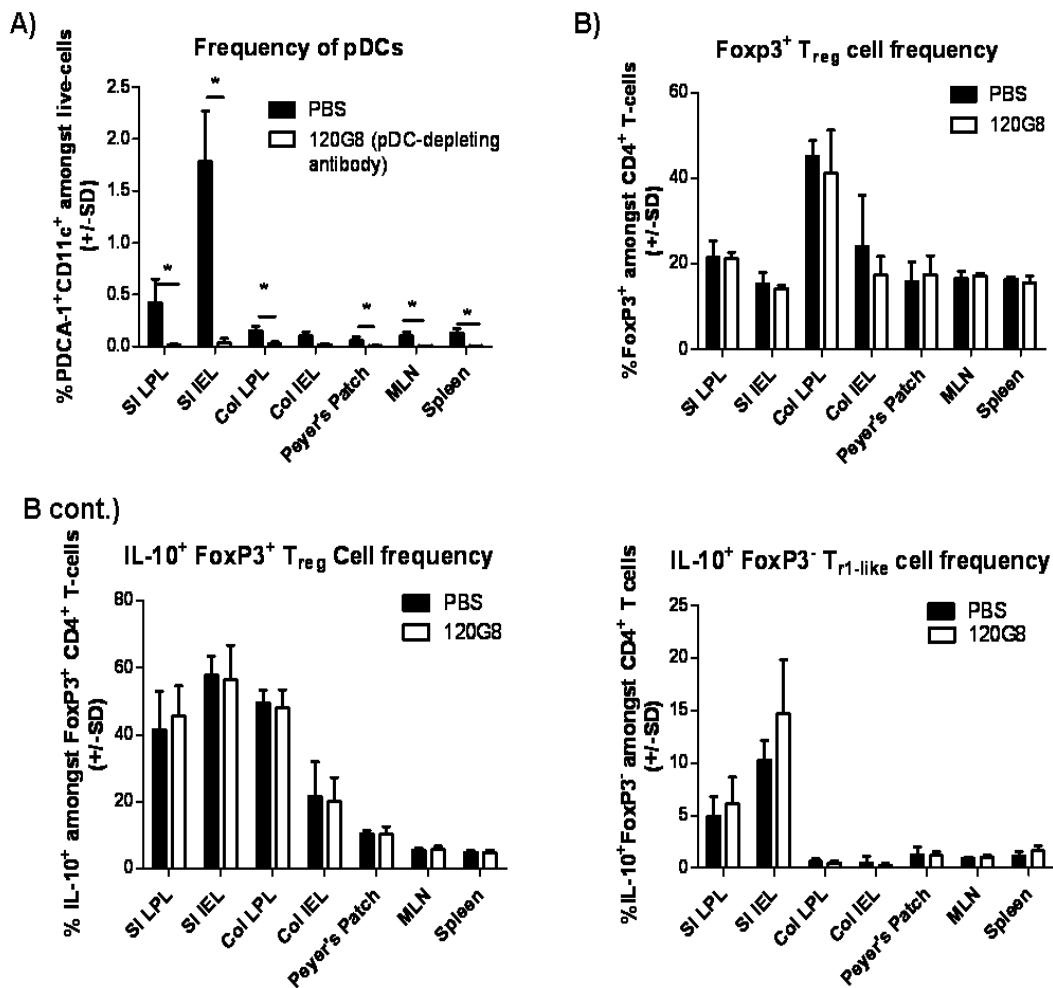


Figure 5.9 Depletion of plasmacytoid DCs (pDCs) has no effect on IL-10 production by intestinal CD4⁺ T-cells

FoxP3^{huCD2}IL-10^{GFP/+} mice were treated with 150µg 120G8 antibody by i.p. injection every other day for two weeks

A) The proportion of pDCs (as defined by PDCA-1⁺CD11c⁺) after treatment with 120G8 pDC-depleting antibody or PBS control. Statistical significance determined by Mann Witney test (*p>0.05)

B) The proportion of FoxP3⁺ T_{reg} cells, IL-10⁺ FoxP3⁺ T_{reg} cells or IL-10⁺ T_{r1-like} cells as determined by flow cytometry pre-gating on CD4⁺ TCR-β⁺ cells. Lack of statistical significance was determined using a Student's *t*-test.

Data represent the mean proportions of different cell types from n=4 biological replicates from one experiment.

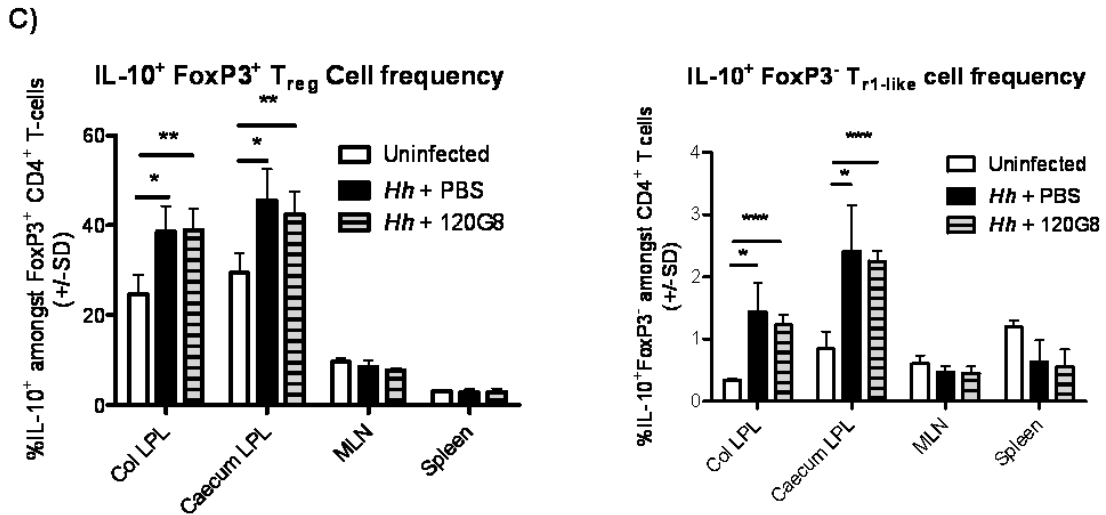


Figure 5.9 cont. pDC depletion has no effect on the induction of IL-10 production by CD4⁺ T-cells following *Helicobacter hepaticus* infection

C) The proportion of IL-10⁺ FoxP3⁺ T_{reg} cells or IL-10⁺ T_{r1-like} cells as determined by flow cytometry in mice infected with 1x 10⁸ *H. hepaticus* (*Hh*) bacteria on 3 consecutive days after the first two injections of 120G8 antibody. 120G8 injections were then continued for 14 days prior to analysis. Statistical significance determine using a Student's *t*-test.

Data represent the mean proportion of different cell types from n=3-5 biological replicates from one experiment.

It should be noted that the antibody used to identify pDCs and the antibody used to deplete pDCs both recognise the same cell surface protein (PDCA-1/BST-2). Although this combination of antibodies has been used to determine depletion of pDCs in the literature [467], we can not be certain that our “depleting” antibody is not blocking the epitope required for identification. Therefore further experiments are required to determine the efficacy of our depletion protocol.

5.2.9 The IL-27R α is expressed at high levels on colonic IL-10⁺ CD4⁺ T cells.

IL-27 is a member of the IL-12 family of cytokines and has been strongly linked to the induction of IL-10, primarily from FoxP3⁻ Tr1-like cells *in vitro* [298-303, 468]. Furthermore, polymorphisms in a locus containing the gene for IL-27p28 have been associated with early-onset IBD, which intriguingly is the same condition in which mutations in the IL-10R have also been uncovered [469]. We therefore decided to analyse the expression of the IL-27R on IL-10⁺ CD4⁺ T cells in the

intestine. The IL-27R is formed of two subunits; a specific IL-27R α chain and GP130, which can also form other heterodimeric cytokine receptors such as for the IL-6R family. Interestingly we found that the majority of CD4⁺ T cells in the colon appeared to express the IL-27R compared to a minority of CD4⁺ T cells in the MLN consistent with the elevation of the IL-27R expression following T cell activation (**Figure 5.10A**). Furthermore, in the colon, IL-10⁺ CD4⁺ T cells were clearly enriched for IL-27R expression compared to IL-10⁻ CD4⁺ T cells (**Figure 5.10A-B**). A similar, but less marked effect was also observed for colonic FoxP3⁺ CD4⁺ T cells. We also analysed expression of the IL-27R α chain on CD4⁺ T cells following *H. hepaticus* infection. Interestingly, IL-27R α was expressed on the IL-10⁺ CD4⁺ T cells in the colons that were induced by *H. hepaticus* infection. Furthermore, expression of the IL-27R α chain on colonic CD4⁺ T cells in general appeared elevated following *H. hepaticus* infection as measured by the MFI of anti-IL-27R α staining (**Figure 5.10C**). These results therefore highlight IL-27 as a cytokine of particular interest in the control of IL-10 producing CD4⁺ T cells in the intestine.

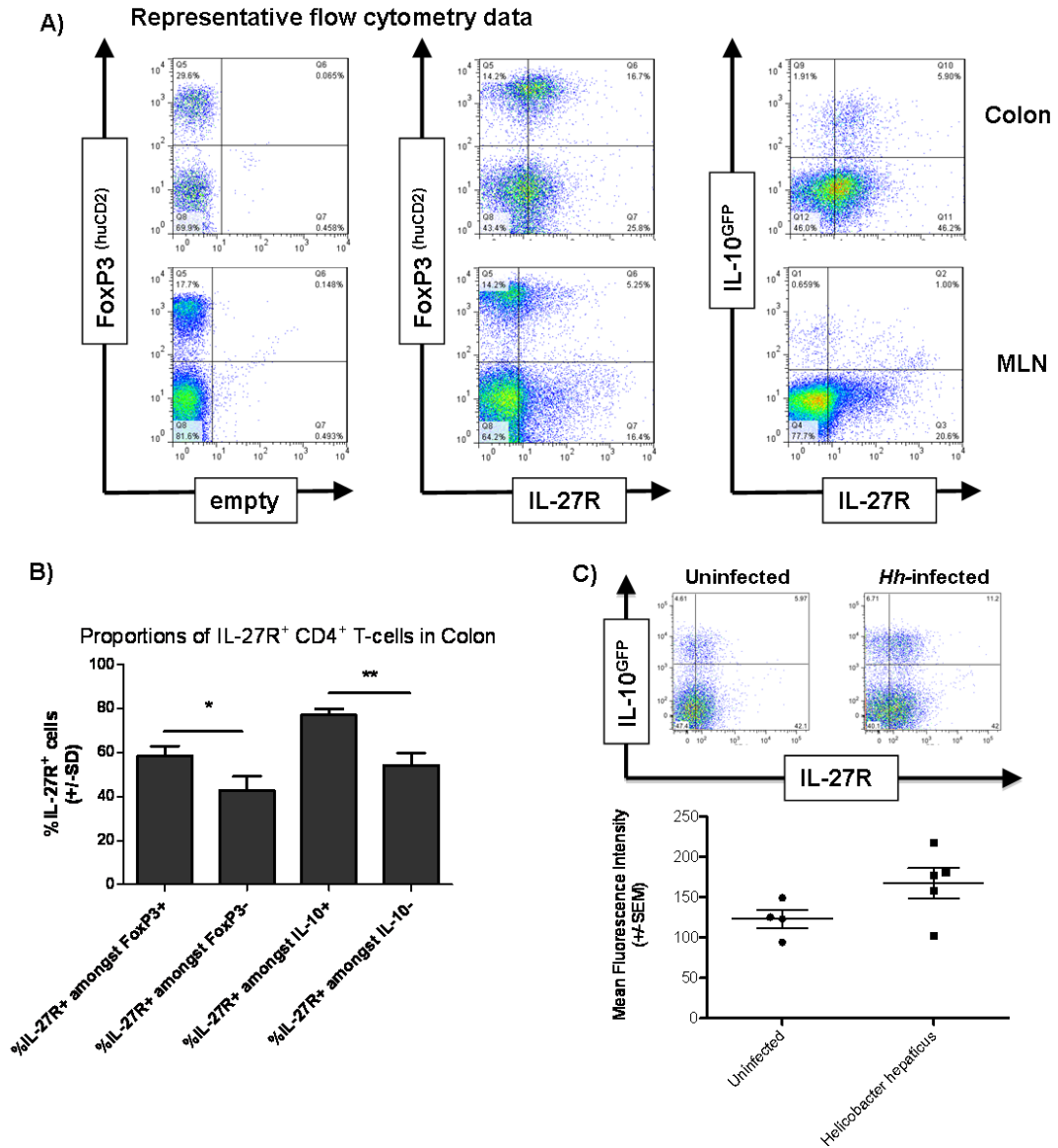


Figure 5.10: IL-27R is expressed at high levels on IL-10⁺ CD4⁺ T-cells in the colon.

A) Single cell suspensions were generated by collagenase digest of colon or MLN of FoxP3^{huCD2}IL-10^{GFP/+} mice. The relative expression of IL-27R on FoxP3⁺ T-cells or IL-10⁺ T-cells is shown by pre-gating on CD4⁺ cells.

B) Enrichment of IL-27R amongst different populations of FoxP3⁺, FoxP3⁻, IL-10⁺ and IL-10⁻ CD4⁺ cells was determined. Statistical significance was determined using a Student's *t*-test.

Data represents the mean of 3 biological replicates from one experiment.

C) IL-27R staining of colon LPLs isolated from uninfected FoxP3^{huCD2}IL-10^{GFP/+} mice or mice infected for 14 days with *Helicobacter hepaticus*. All plots shown are pre-gated on CD4⁺ TCR-β⁺ cells. The mean fluorescent intensity of anti-IL-27R-PE binding to cells is shown graphically. Each data point represent an individual biological replicate from one experiment

5.2.10 The expression of IL-12 family members in the colon following *Helicobacter hepaticus* infection

Our hypothesis that IL-27 was important for controlling IL-10 production by intestinal CD4⁺ T cells led us to analyse the expression of IL-12 family members in colonic tissue following *H. hepaticus* infection when the expression of IL-10 is clearly elevated (**Figure 5.11A**). IL-27 is composed of two subunits, IL-27p28 which is generally considered to be the main inducible element, and EBI-3, which is expressed more widely [292, 470]. Interestingly, and consistent with our hypothesis there was a trend towards elevated IL-27p28 and to a lesser extent EBI-3 over the course of infection correlating with the elevation in IL-10 expression (**Figure 5.11A**). However, the extremely low level of expression detectable in whole colon makes it difficult to determine the true significance of this increase. In addition, IL-23p19 was elevated by day 14 post infection although this was after the levels of IL-10 had begun to increase making it less likely that IL-23 is regulating IL-10 expression in response to *H. hepaticus*. We observed no increase in the expression of IL-12p35 following infection.

Given the correlation between IL-10 and IL-27 following *H. hepaticus* infection, we investigated whether the microbiota itself, which induces IL-10, also induces IL-27. Comparing the expression of IL-10 in whole colon pieces dissected from Germ-free, altered Schaedlers flora (ASF)-colonised or conventionally housed B6 mice, showed that IL-10 expression was reduced in the absence of the microbiota consistent with our data following antibiotic treatment. An analysis of IL-27p28 in the same conditions showed a trend towards an elevation in IL-27p28 expression in conventionally housed mice again suggesting that IL-27p28 and IL-10 expression might correlate (**Figure 5.11B**). However, it should be noted that the

sensitivity of analysing IL-10 and IL-27p28 expression in this way is extremely low and further experiments are required, particularly on enriched leukocyte populations, to confirm this correlation.

5.2.11 IL-27p28 expression is significantly up-regulated during *Helicobacter hepaticus*-induced inflammation.

IL-27, along with other IL-12 family members, also has reported pro-inflammatory roles [470, 296, 297, 471, 229]. We therefore analysed the expression IL-10 and IL-27p28 during the development of colitis induced by IL-10R blockade concurrent with *H. hepaticus* colonisation. Interestingly, we observed a marked elevation in IL-27p28 expression in colon tissue in the first two weeks following disease induction during which time sizeable cell infiltration occurs (including CX3CR1^{int} cells described in chapter 3) (**Figure 5.12A**).

In addition, analysis of IL-10 expression in *H. hepaticus* infected FoxP3^{huCD2}IL-10^{GFP/+} mice during IL-10R-neutralisation showed that IL-10 production remains elevated despite the on-going inflammation and was in fact enhanced in FoxP3⁻ Tr1-like cells (**Figure 5.12B**). This indicates that the increase in IL-10 production by intestinal CD4⁺ T cells in response to *H. hepaticus* is not dependent on IL-10 itself and also suggests that the pathway is not inhibited by the activation of inflammatory responses in the intestine.

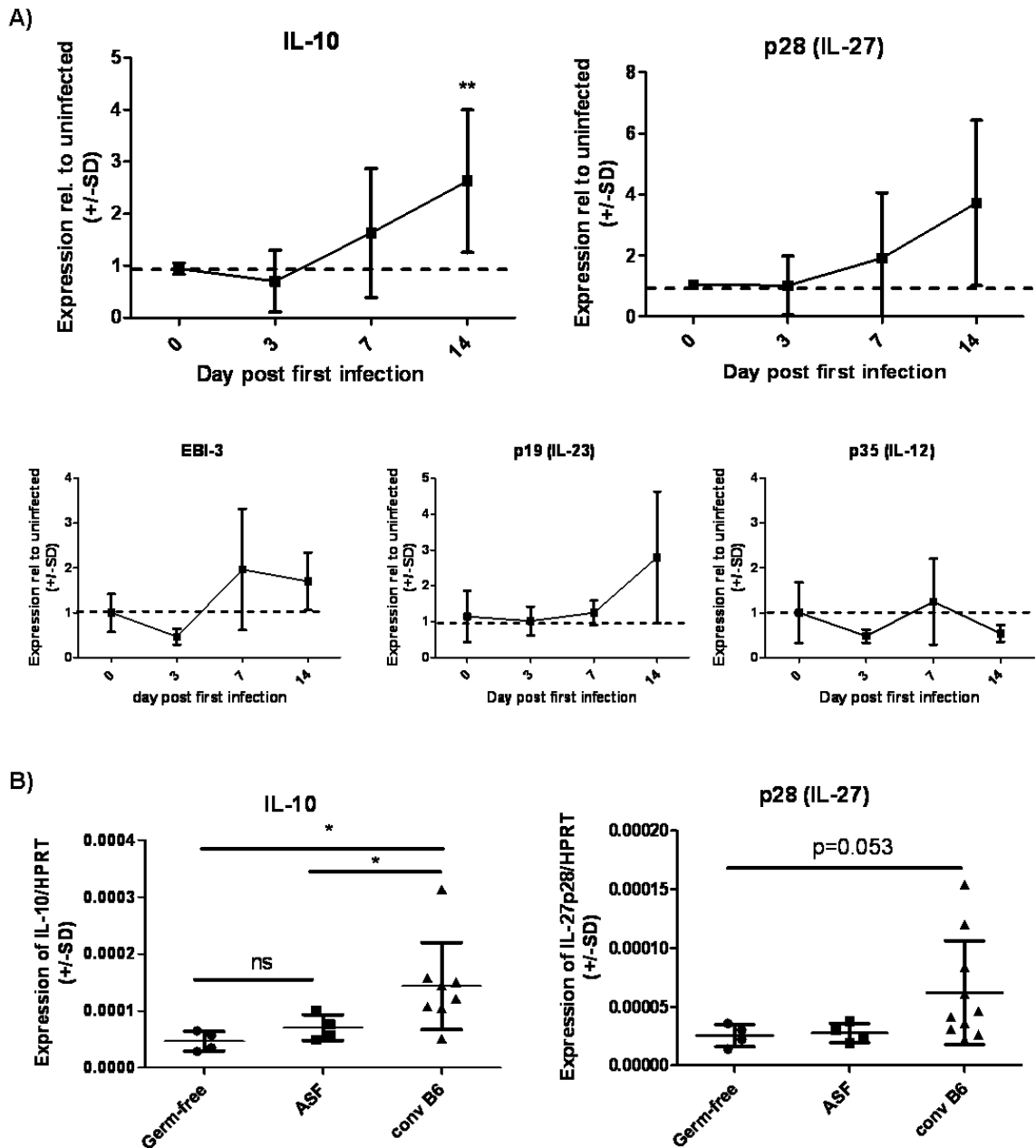


Figure 5.11: IL-10 and IL-27 expression are elevated after *Helicobacter hepaticus*-infected mice and reduced in germ-free mice

A) To assess expression of IL-12 family cytokines following *Helicobacter hepaticus* infection, RNA was extracted from pooled pieces of proximal, distal and mid colon of mice either prior to infections (day 0) or at day 3, day 7 and day 14 post infection with *Helicobacter hepaticus*. The relative mRNA expression of specified cytokines was determined by Q-PCR controlled to HPRT.

Data represent the mean of n=4-6 biological replicates from one experiment. Subsequent experiment by J. Bollrath yielded similar results.

B) To assess the effect of the microbiota on IL-10 and IL-27p28 expression RNA was extracted from pooled pieces of proximal, distal and mid colon harvested from germ-free mice, mice colonised with a limited flora (ASF) (M. Geuking, Bern) or conventionally housed C57Bl6 controls. Expression was determined by Q-PCR relative to HPRT.

Each data point represents a single biological replicate from one experiment.

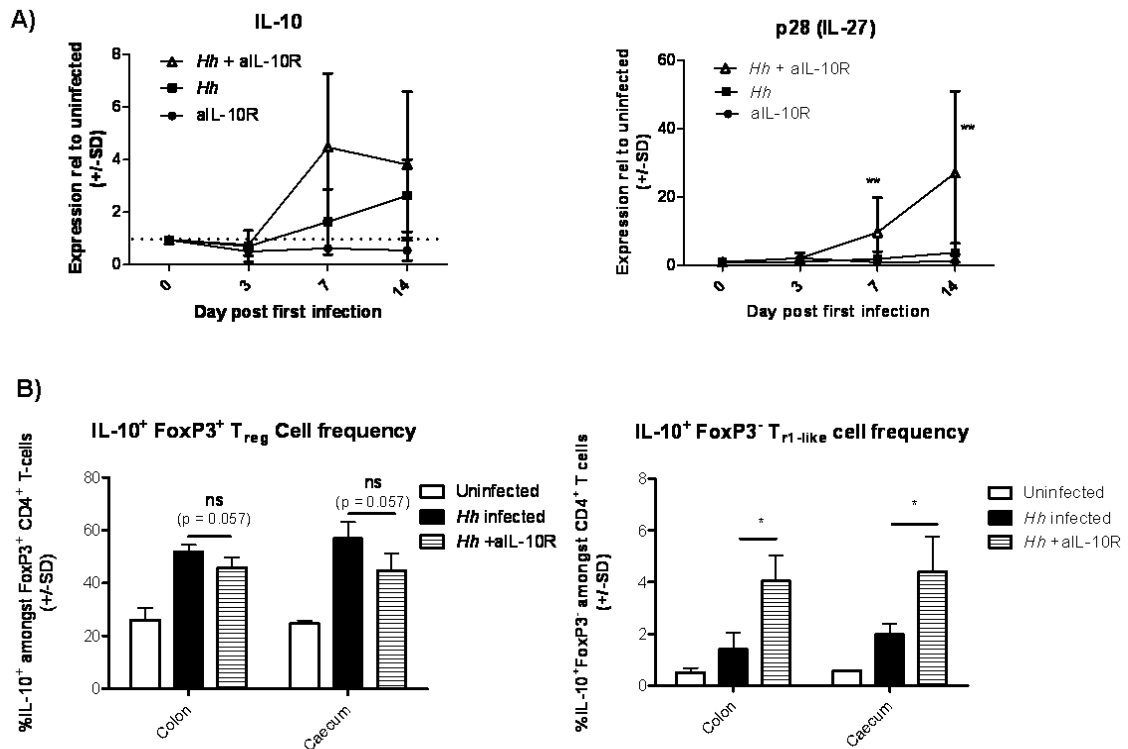


Figure 5.12 IL-27 and IL-10 expression are up-regulated during *H. hepaticus* induced inflammation

A) To assess cytokine expression during the development of *Helicobacter hepaticus*-induced inflammation mRNA was extracted from pooled pieces of proximal, distal and mid colon from mice either prior to disease (Day 0, n=6) or at day3 (n=4), day 7 (n=4) or day 14 (n=4) after infection with *Hh* and i.p injection of anti-IL-10R antibody (1mg/mouse) on day 0 and day 7. Cytokine expression was determined by Taqman Q-PCR.

Data represent the mean of n = 4-6 biological replicates from one experiment.

B) To assess IL-10 production by intestinal CD4⁺ T-cells inflammation was induced as described above in FoxP3^{hu}CD2IL-10^{GFP/+} mice (n=5). At day 14, single cell suspensions generated from colonic and caecal LPLs and compared to age-matched uninfected or *Helicobacter hepaticus* infected (n=4) non-inflamed controls (n=2). IL-10 expression by FoxP3⁺ Treg and FoxP3⁻ Tr₁-like cells was analysed by flow cytometry.

Data represent the mean of n= 2-4 biological replicates from one experiment.

Statistical significance was determined using a Mann-Witney test. (*p<0.05, **p<0.01)

5.2.12 IL-10 inhibits *Helicobacter hepaticus* induced IL-27p28 expression in DCs *in vitro*

The suggestion that IL-27p28 expression is induced by *H. hepaticus* infection *in vivo* and that expression of IL-27p28 was substantially increased by blockade of IL-10 signalling led us to investigate whether this could be a cell intrinsic effect in DCs. In a preliminary experiment, stimulation of BMDCs *in vitro* for 24hrs with *H. hepaticus* (MOI 2 or MOI 10) resulted in a 10 fold increase in IL-27p28 mRNA

expression (**Figure 5.13A**). This was equivalent to stimulation with Flagellin, but 10 fold less than the level of IL-27p28 expression observed after stimulation with LPS. Interestingly, and consistent with a direct role for IL-10 inhibiting IL-27p28 expression in DCs, this up-regulation could be blocked by addition of IL-10 to the cultures 24hrs prior to exposure to *H. hepaticus* (**Figure 5.13B**). Reassuringly, no inhibition was observed if IL-10R signalling was simultaneously blocked and there was evidence of a further increase in IL-27p28 expression if endogenous IL-10 production was prevented by using BMDCs generated from IL-10^{-/-} mice. Therefore, *H. hepaticus* is able to directly induce IL-27p28 expression in BMDCs and this is regulated by IL-10 *in vitro*.

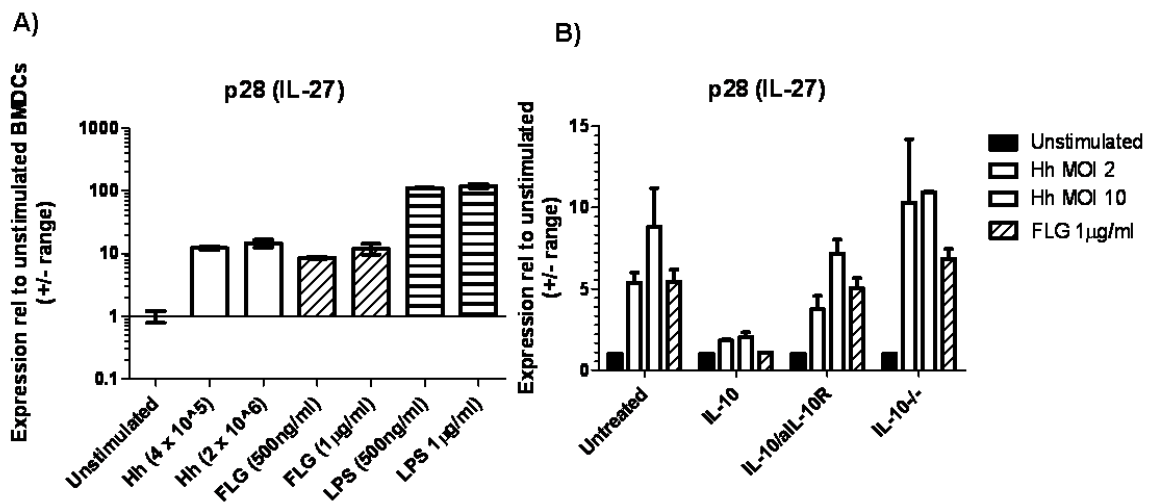


Figure 5.13: Preliminary experiment: IL-27 is induced in BMDCs in response to Hh and is regulated by IL-10 *in vitro*.

- A) DCs (CD11c⁺MHCII⁺) were generated by culturing mouse bone-marrow cells with GM-CSF (20ng/ml) for 6 days. 2 x 10⁵ DCs were then treated with *Helicobacter hepaticus* (*Hh*) at a rough MOI of 2 (4 x 10⁵ cells per well) or an MOI of 10 (2 x 10⁶), Flagellin (FLG) or LPS (at specified concentrations) for 24 hours. RNA was then extracted and the expression of IL-27p28 determined by Q-PCR.
- B) Mouse BMDCs were pre-treated with IL-10 for 24 hours, in the presence or absence of antiIL-10R antibody, and compared to untreated BMDCs or IL-10^{-/-} BMDCs to control for endogenous IL-10 production. BMDCs were then stimulated with *Helicobacter hepaticus* or flagellin as described above and the expression of IL-27p28 determined by Q-PCR.

Data represent the mean +/- range of n=2 biological replicates from one experiment.

5.3 Discussion

The diverse community of bacteria in the mammalian intestines plays a crucial role in the development and proper function of the immune system [172]. In particular emerging evidence has linked changes in the composition of the microbiota with the progression of pathology in various inflammatory disease models, including IBD [190, 278], arthritis [372], diabetes [376], asthma [374, 375] and CNS inflammation [373]. Therefore, understanding the specific immunological pathways responsive to the microbiota could have profound consequences. We hypothesised that the composition of the microbiota might alter Treg cell function and so influence susceptibility to auto-inflammatory immune reactions.

Our approach involved analysing the impact of a broad spectrum antibiotic treatment protocol on FoxP3⁺ iTreg cell induction, overall FoxP3⁺ Treg cell frequency and IL-10 production. It is important that we follow up the findings presented here with a detailed analysis of the impact that antibiotic treatment has on the microbial community in our colony. Without this it is impossible to properly integrate our work fully with the many recent studies, discussed below, that have used a combination of germ-free, antibiotic, monocolonisation and infection models to link different bacterial species with different immune pathways. However, establishing technology to allow high throughput 16srRNA and microbial DNA sequencing as well as the means to handle the large data sets generated poses a considerable challenge. Our intention is to establish this technology whilst also confirming these findings in germ-free systems, although again establishing collaborations to allow this has not proven straightforward. Therefore taking into account the limitations of the antibiotic-treatment protocol we now discuss our findings below in the context of emerging data.

5.3.1 The influence of the microbiota: Controlling FoxP3⁺ Treg cell frequency

Consistent with recent studies comparing germ-free and SPF mice [250, 376, 458] we found no impact of antibiotic treatment on the overall proportion of FoxP3⁺ Treg cells in the small intestine, Peyer's patches or MLN. In addition, we found no impact of antibiotic treatment on the overall proportion of FoxP3⁺ Treg cells in the colon. In contrast to this, other studies have observed a decrease in FoxP3⁺ Treg cells in the colon of germ-free mice compared to conventionally housed mice [472]. Furthermore, colonisation of germ-free mice with a conventional microbiota or with ASF causes an accumulation of colonic FoxP3⁺ Treg cells [472, 473]. Similarly, colonisation with SFB alone induces a mild up-regulation of FOXP3 gene expression (1.5 fold) in the colon [226]. One interpretation of the data which resolves this conflict is that there is a necessary accumulation of FoxP3⁺ Treg cells in the colon upon bacterial colonisation, but that, once generated, these FoxP3⁺ Treg cells do not require on-going bacterial stimulation to maintain FoxP3 expression. Alternatively, there may be underlying differences in the composition of the microbiota in the different mouse colonies. It was indicated that a key inducer of FoxP3⁺ Treg cell accumulation in the colon is a cluster of *Clostridium* species [472]. It will be important to determine whether a similar cluster is present in the microbiota of our facility.

We also analysed the impact of antibiotic treatment specifically on the generation of iTreg cells by orally administered antigen. Interestingly, we found a slight increase in the proportion of FoxP3⁺ Treg cells and a slight decrease in the CD4⁺ T cell proliferation in antibiotic treated animals suggesting that the microbiota acts as an adjuvant to inhibit iTreg cell generation during homeostasis. This is

consistent with evidence that microbiota-derived DNA inhibits Treg cell induction in the intestine in a TLR9-specific manner [359]. Our study was carried out using mice on a Balb/c background which are less resistant to weight loss and dehydration caused by antibiotics than C57Bl/6 mice. Although this could be ameliorated by providing soft chow we have obtained similar OVA₃₂₃₋₃₃₉-specific (OT-II) mice on a C57Bl/6 background to reduce the risk that this finding is caused by side effects of antibiotic treatment and so this finding awaits further confirmation.

5.3.2 The influence of the microbiota: Controlling CD4⁺ T cell IL-10-production

By crossing a FoxP3^{huCD2/huCD2} reporter mouse, which expresses human CD2 under control of the FoxP3 promoter [461], with an IL-10^{GFP/+} reporter mouse, which expresses GFP under control of the IL-10 promoter [405], we were able to map both FoxP3⁺ Treg cells and FoxP3⁻IL-10⁺ Tr1-like cells in the intestine (M. Barnes, A. Johnson, J. Bollrath, manuscript in preparation). Consistent with previous reports, we observed an enrichment of FoxP3⁺IL-10⁺ Treg cells in the intestinal lamina propria [263, 289, 405]. We also observed an enrichment of FoxP3⁻IL-10⁺ Tr1-like cells within small intestine, again similar to previous reports [289]. However, the proportion of Tr1-like cells identified in the small intestine in our study was substantially lower than that observed by Maynard *et al* who used a different dual reporter system reliant on the BAC-transgenic expression of Thy1.1 under control of the IL-10 promoter. It is conceivable that a BAC-transgene is not under the same level of control, either epigenetically or by transcriptional co-repressors [279], as the endogenous IL-10 gene into which the IRES^{GFP} knock-in was inserted [405]. It was noted by Maynard *et al* that the level of IL-10 produced

by IL-10^{Thy1.1+} CD4⁺ T cells was low directly *ex vivo* , but increased dramatically after stimulation leading the authors to suggest that Thy1.1 expression was identifying cells “competent” for IL-10 production, but that they may not in fact be “actively producing” IL-10 *in vivo*. Similar analysis of IL-10^{GFP+} and IL-10^{GFP-} cells isolated from our reporter mice may help to indicate whether there are intrinsic differences between the reporter systems that could explain the different proportions of intestinal FoxP3⁻IL-10⁺ Tr1-like cells observed between these studies. Alternatively, given the remarkable sensitivity of IL-10 expression to alterations in the microbiota, it is possible that the differences in FoxP3⁻IL-10⁺ Tr1-like cells are caused by differences in the composition of the microbiota.

Strikingly, we found that antibiotic treatment caused marked ablation of IL-10 production by both FoxP3⁺ Treg cells and FoxP3⁻ Tr1-like cells not just in the intestine but also in tissues peripheral to the gut. This is consistent with recent reports showing a reduction in IL-10 expression in the colon of germ-free mice compared to conventionally housed mice [226, 276, 472]. The systemic effect on IL-10 production observed following antibiotic treatment is interesting as it might indicate a potential mechanism by which the microbiota modulates the severity of inflammatory diseases developing in locations away from the intestine such as joints [372] or the CNS [373]. However, it is difficult to separate out potential systemic side effects of antibiotic treatment. Therefore determining whether there are systemic changes in IL-10 production in germ-free mice or after colonisation of germ-free mice would represent important confirmation of our finding

Increasingly specific bacterial species within the microbiota have been linked to specific cytokine responses. For example, SFB has been strongly linked with Th17 cell induction [201, 226]. In fact, SFB colonisation also has a mild effect IL-

IL-10 expression in the colon [226] although this has not been observed in all studies [201, 472]. Interestingly, the colonisation of germ-free mice with a cluster of *Clostridium* species or with ASF, which contains a *Clostridium* component, causes the accumulation of FoxP3⁺IL-10⁺ Treg cells in the colon [472, 473]. It remains to be seen whether a similar cluster of *Clostridium* species in the microbiota are promoting IL-10 expression in our colony. As *Clostridia* are particularly sensitive to vancomycin treatment, and vancomycin alone was able to reduce IL-10 expression in the study of Atarashi *et al*, analysis of IL-10 production following treatment of our mice with single antibiotics may help to indicate particular families of bacteria promoting IL-10 production in our colony.

In the context of the general microbiota we found that the bacteria *H. Hepaticus* significantly up-regulated IL-10 production by both FoxP3⁺ Treg cells and FoxP3⁻ Tr1-like cells. This is similar to effects observed following colonisation of mice with the human commensals *Bacteriodes fragilis* or *Faecalibacterium prausnitzii* [276, 385, 472]. Interestingly, colonisation with either of these bacteria offers therapeutic benefit in mouse models of colitis and it will be interesting to see whether *H. hepaticus* colonisation can offer similar, context dependent, benefit. *B. Fragilis* mediates its effect through a carbohydrate, polysaccharide A (PSA). We observed that feeding heat killed *H. hepaticus* did not induce IL-10 suggesting a requirement for live bacteria rather than its constituents alone. However, heat killing bacteria in this fashion could cause some denaturing of constituent proteins and carbohydrates therefore it is important to repeat this experiment using bacteria killed by UV-irradiation. It also remains unclear whether *H. hepaticus* acts directly to induce IL-10 production or indirectly by altering the microbial environment in favour of other “IL-10 inducing” populations in the microbiota such as the

Clostridium cluster. In favour of indirect effects, studies analysing the inflammatory potential of *H. hepaticus* have suggested a failure to promote inflammation in germ-free animals [474] although this conflicts with other reports [475] and maybe due to the relatively short time frame of the study. Rather it has been suggested that *H. hepaticus* requires the presence of at least one other species within the colon to induce a colitic response [476]. We have established a collaboration to colonise germ-free mice with *H. hepaticus* alone or in combination with ASF to determine whether *H. hepaticus* alone is sufficient for the induction of IL-10-producing CD4⁺ T cells.

5.3.3 The mechanism controlling IL-10 production in the intestine: Potential dual role for IL-27

Consistent with recent data we found that control of IL-10 production by the microbiota was independent of MyD88 [472]. However, FoxP3⁺ Treg cell accumulation in the colon following ASF colonisation is ablated in MyD88^{-/-}TRIF^{-/-} mice [473]. Therefore, these data collectively implicate TRIF-signalling in IL-10 production or alternatively, there might be considerable redundancy so that any TLR-stimuli can suffice. *B. Fragilis* induces IL-10 in a MyD88-TLR2-dependent fashion and the induction of inflammation by *H. Hepaticus* is MyD88-dependent, therefore it was a surprise to find that IL-10 induction by *H. Hepaticus* was independent of MyD88. Analysis of IL-10 expression in TRIF^{-/-} and MyD88^{-/-}TRIF^{-/-} mice will help to resolve the requirement for PRRs in the control of IL-10 production by the microbiota.

The preliminary data presented here highlight IL-27 as a potential candidate for the control of IL-10 production by intestinal CD4⁺ T cells. In particular we show elevated IL-27R expression on IL-10⁺ CD4⁺ T cells in the colon, and correlations

between IL-27 and IL-10 expression in colonised mice compared to germ-free mice and following *H. Hepaticus* infection. This is in accordance with the described role for IL-27 in promoting IL-10 production by FoxP3⁻ Tr1-like cells and, to a lesser extent, FoxP3⁺ Treg cells *in vitro* [298-303, 468] (J. Bollrath F. Powrie, unpublished data). However, the sensitivity of analysing cytokine expression in mRNA from pieces of whole colon is extremely low and therefore this data should be interpreted with caution. For example, we were unable to detect a significant elevation in IL-10 in ASF colonised mice compared to germ-free mice, even though literature suggests at the level of purified CD4⁺ T cells such elevation in IL-10 expression is quite clear [473]. Therefore, it is important to repeat these experiments to analyse IL-27 expression in better defined cell populations, especially myeloid cells which are thought to be the major producers of IL-27 *in vivo* [291, 477].

Interestingly, IL-27p28 expression in human DCs is strongly promoted by gram negative bacteria, but much less so by gram positive bacteria suggesting differential regulation by different bacteria strains [478]. Furthermore, expression of IL-27p28 in bone marrow-derived macrophages is dependent on TRIF-signalling and the TRIF-dependent cytokines, type I IFNs, rather than MyD88-signalling [479, 480], in alignment with the pathways we hypothesise are responsive to the microbiota in our system [473, 481]. Interestingly, we observed that *H. Hepaticus* was able to induce IL-27p28 expression in BMDCs *in vitro*. Therefore, deriving from our preliminary data, we hypothesise that bacterially-driven IL-27 production promotes IL-10 expression in the intestine as a mechanism for maintaining tolerance. This hypothesis is supported by evidence that administration of exogenous IL-27 ameliorates TNBS-induced colitis [482]; similar to effects

observed in models of arthritis [483], central nervous system inflammation [301], and delayed-type hypersensitivity responses [484].

Conversely, IL-27 can also have pro-inflammatory effects, in particular in promoting Th1 cell differentiation [293-295]. Interestingly, we also observed a significant up-regulation in IL-27 expression during the onset of inflammation resulting from *H. hepaticus* colonisation and concurrent IL-10R blockade. This correlated with the infiltration of monocyte-derived CX3CR1^{int} cells described previously and similar infiltrating cell populations have been shown to produce IL-27 in the inflamed joints of rheumatoid arthritis patients [485]. Intriguingly, a similar up-regulation of IL-27p28 expression was observed in another bacterially-driven IL-10-deficient model (IL-10^{-/-} mice) [486]. The up-regulation in IL-27p28 expression may represent a feedback loop to enhance IL-10 production and so limit the inflammatory response. In accordance with this IL-10⁺ CD4⁺ T cells are increased during *H. hepaticus*-induced inflammation despite blockade of IL-10 signalling and the on-going inflammatory response. Alternatively, during colitis IL-27 may play a pro-inflammatory role. This hypothesis is supported by studies in other models of colitis induced by DSS-treatment [296], helminth-infection [487], oxazalone-treatment [488] or T cell transfer [297] where IL-27-deficiency or IL-27R-deficiency reduced the severity of disease.

Resolving these contrasting effects of IL-27 is difficult however it seems likely that the presence of co-factors, such as AHR ligands [298] and the kinetics of CD4⁺ T cell polarisation likely play a key role. For example, IL-27 signalling on pre-polarised Th1, Th2 or iTreg cells can promote IL-10 production [300, 302], (J. Bollrath, personal communication), whereas IL-27 signalling upon T cell activation causes early up-regulation of the IL-12R, inhibition of iTreg cell differentiation and

enhancement of Th1 cell differentiation [291, 294, 295, 297, 489]. Hypothetically, CD4⁺ T cells infiltrating the lamina propria during inflammation are likely to be more recently activated and, as IL-27 expression is increased, are more likely to encounter “early” IL-27 signalling thus perpetuating an inflammatory response.

Given these dual pro- and anti-inflammatory roles for IL-27 it is expected that tight regulation of IL-27 production *in vivo* would be advantageous. Here we show that *in vitro* BMDC expression of IL-27p28 in response to *H. hepaticus* could be inhibited by exogenous IL-10. We propose the presence of a feedback loop *in vivo* whereby IL-27 induces IL-10 which feeds back to limit IL-27 expression and thus maintain mutualism (**see figure 5.14**). Analysis of IL-27 expression in DCs or macrophages from IL-10^{-/-} mice or STAT3^{-/-} mice (through which IL-10 signals to prevent the development of inflammation) might indicate whether similar regulation is evident *in vivo*. Furthermore, the analysis of CD4⁺ IL-10 expression and inflammatory responses following *H. hepaticus* infection in IL-27Rα^{-/-} or EBI-3^{-/-} mice will allow dissection of the relative contribution of IL-27 in each setting to be determined. These strains are currently being obtained.

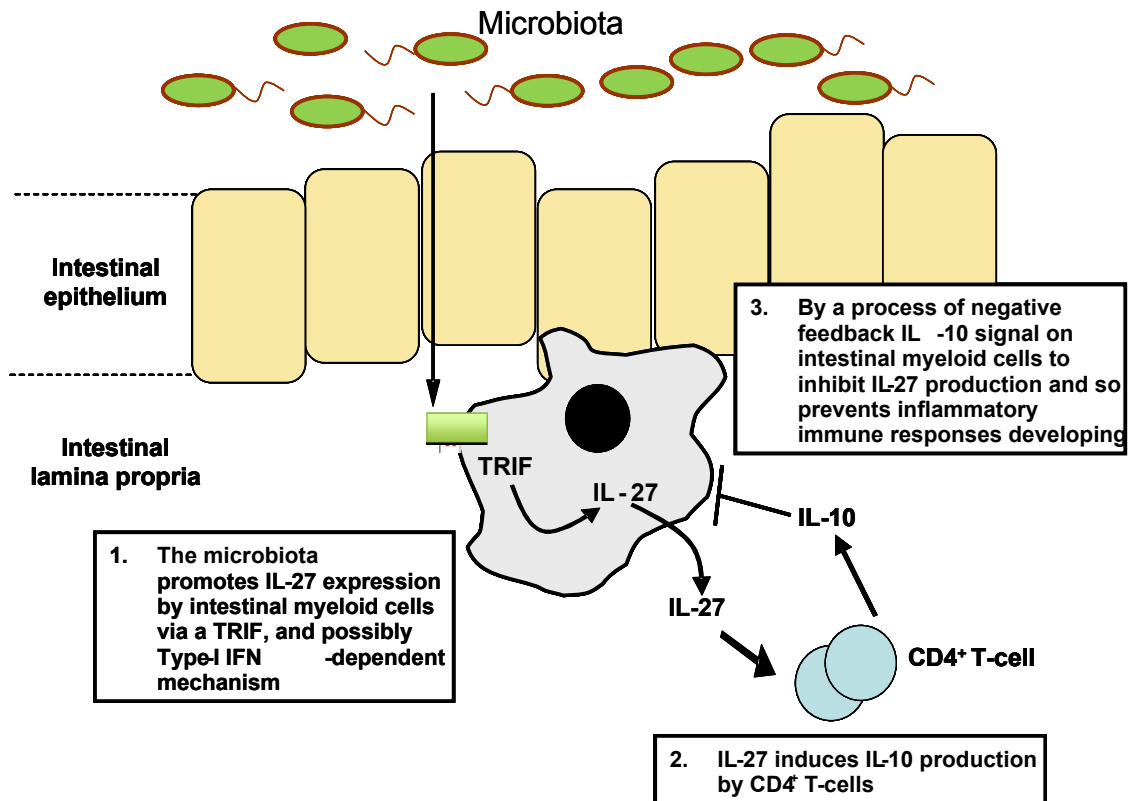


Figure 5.14 Hypothetical model for the interplay between the commensal flora, IL-27 and IL-10 in the regulation of immune responses in the intestine

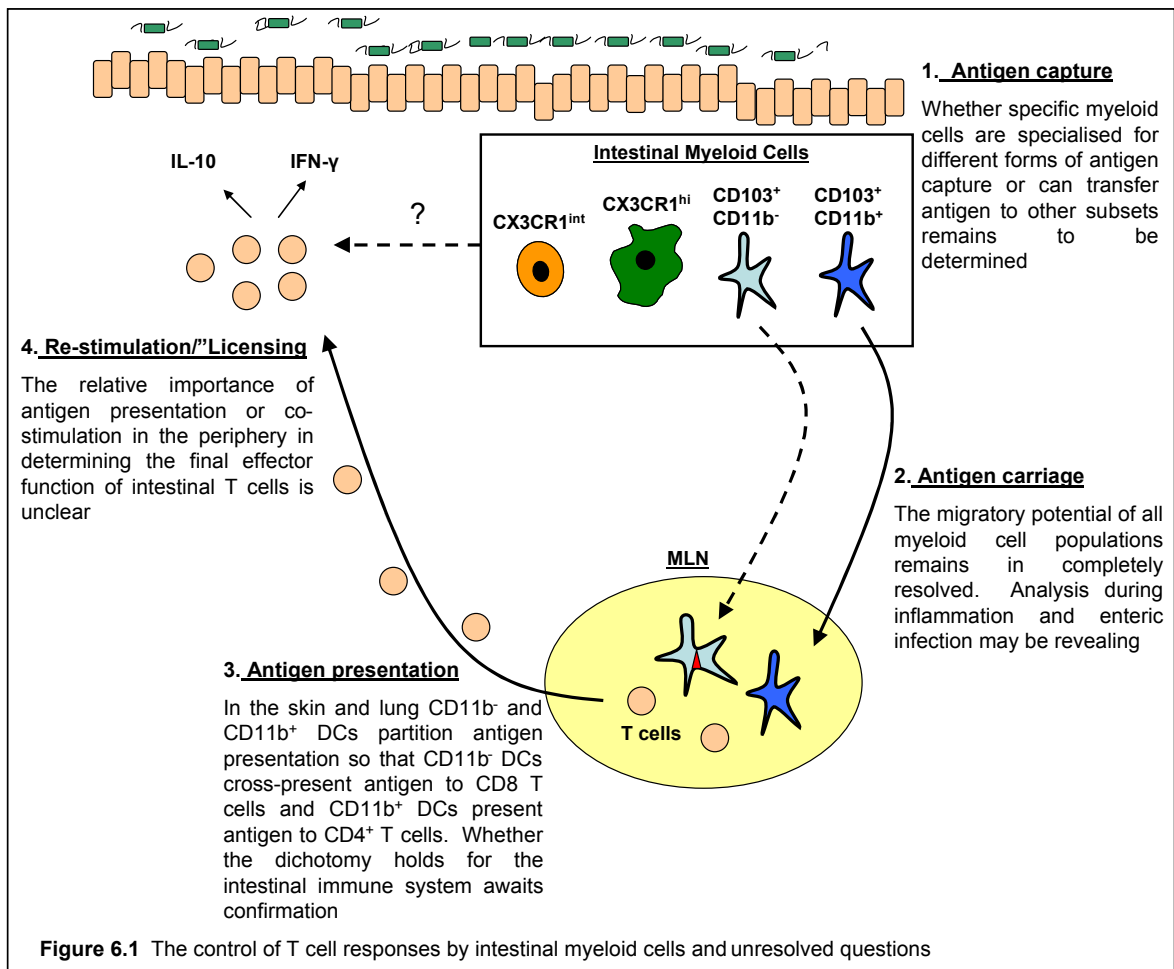
Chapter 6

General discussion

6.1 Overview

Detailed discussion sections critically evaluating the data presented and relating it to directly relevant literature were included in the results chapters. The purpose of this general discussion section is to more broadly assess how this thesis fits into a wider conceptual framework and to indicate the immediate future experiments which are recommended to extend this work further.

The layout of this section has been deliberately constructed to mirror the progression of results chapters. However, it is important to consider that intestinal bacteria, DCs and IL-10-producing Treg cells are all part of an integrated system of antigen presentation of the type described in the general introduction (**see figure 6.1**) and therefore all the work presented in this thesis is intrinsically linked. Elegant work on similar systems in the skin has begun to probe in more detail the mechanics of the DC and T cell responses to subcutaneously applied antigen [98, 490]. The work established here, where the intestinal DC subsets were further characterised and where the oral application of antigen or intestinal bacteria was used to induce an iTreg cell or IL-10 response, offers similar opportunities to probe the mechanics of DC and T cell responses specifically in the intestine. The detail of experiments that the author suggests would address this overall aim is highlighted at the end of each of the following sections.



6.2 The functional role of intestinal DCs

Described in this thesis and in recent publications is a description of the character of the myeloid cell network in the lamina propria with particular focus on CD103⁺ DCs and CX3CR1⁺ cells. It is apparent that two populations of CD103⁺ DCs (CD103⁺CD11b⁺ and CD103⁺CD11b⁻) and two populations of CX3CR1⁺ cells (CX3CR1^{int} and CX3CR1^{hi}) are present with distinct phenotypes and different patterns of tissue localisation and accumulation (discussed in detail in chapter 3, summarised in **figure 3.15**) [24, 25, 35, 62, 157, 158, 310, 311, 412, 428]. However, the challenge still remains to determine precisely the importance of DCs in the maintenance of intestinal tolerance and in particular how these different

myeloid cell populations co-operate to induce distinct immune responses, such as iTreg cell and IL-10 induction.

In order to address this challenge two strains of CD11cDTR mice have been imported in which diphtheria toxin (DT) can be used to selectively deplete CD11c⁺ cells: CD11cDTR [9] and CD11cCre.iDTR mice [491, 492]. In addition OT-II mice have been crossed to the FoxP3^{huCD2/huCD2}IL-10^{GFP/GFP} dual reporter mice to enable the iTreg and IL-10 responses to a defined dietary antigen to be modelled *in vivo*. Similarly, CBir mice [493] have been obtained in order for the response to a partially-defined commensal bacteria antigen [494, 495] to be modelled *in vivo*. These tools can be combined to directly to assess the impact of DC depletion on the immune response to oral antigen, commensal bacteria or *Helicobacter hepaticus* colonisation either in a tolerance or vaccination setting. The specific immediate experiments suggested are outline below

Immediate future experiments to probe function of intestinal DCs

- Using bone marrow chimeras where CD11cDTR is expressed in the haematopoietic compartment or CD11cCre.iDTR mice, DCs are depleted by DT injection and the effect on FoxP3 and IL-10 expression by CD4⁺ T cells assessed (initially by intracellular cytokine staining) either in *H. hepaticus* infected or control mice. This experiment can be extended to deplete DCs prior to or subsequent to *H. hepaticus* infection to determine the relevance of DCs more specifically in the initiation and/or the perpetuation of the IL-10 response. Finally the addition of a group of mice in which IL-10R antibody is administered concurrent to *H. hepaticus* infection would enable assessment of the relative contribution of DCs to the induction or protection against *H. hepaticus* induced colitis to be assessed. A preliminary version of this

experiment suggested that DC depletion was coupled to substantial granulocyte (CD11b⁺ Ly6C⁺) recruitment and the induction of IFN- γ -producing CD4⁺ T cells and this was evident in both uninfected, *H. helicobacter*-infected and colitic (anti IL-10R treated) mice relative to non DC-depleted control mice. However, it was not entirely clear whether this was due to DC depletion or the result of DT acting as an adjuvant. Therefore, in subsequent experiments it is important for WT \rightarrow WT chimeras or CD11cCre mice to be used as a DT-treated, non DC-depleted control group.

- Given that assessment of IL-10 production by intracellular antibody staining is technically challenging and the sensitivity is low a second experiment is suggested. OT-II.FoxP3^{huCD2⁺}.IL-10^{GFP⁺} CD4⁺ are adoptively transferred into CD11cDTR chimeras or into CD11cCre.iDTR mice and ovalbumin, containing the cognate epitope, is administered orally by gavage to elicit a CD4⁺ T cell response. The depletion of DCs in the recipient mice either prior to or proceeding ovalbumin administration would enable the requirement for DCs in the induction or maintenance of FoxP3^{huCD2⁺} Treg cells and IL-10^{GFP⁺} cells to be assessed without a requirement for intracellular cytokine staining. This experiment could also be modified to administer ovalbumin with oral adjuvant such as cholera toxin or even bacteria such as *H. hepaticus* in order to assess the importance of DCs in the immune response in an oral vaccine setting.

6.3 Specific functions of intestinal CD103⁺ and CX3CR1⁺ myeloid cell subsets

The DC mediated initiation of immune responses in the skin is driven by the coordinated migration of different DC subsets to the draining lymph nodes, antigen presentation to activate naive T cell responses and finally re-stimulation of effector T cells in the periphery [98, 99, 490]. In particular, CD103⁺CD11b⁻ DCs from the

dermis, which closely resemble the CD103⁺CD11b⁻ DCs described in the colon, migrate to cross-present antigen to CD8⁺ T cells [424]. Interestingly, this process is hypothesised to enable cross-tolerance of apoptotic cell derived antigens to be maintained in the skin and the lung [58, 425, 496]. In comparison CD11b⁺ dermal DCs, which do not express CD103, migrate and present antigen to CD4⁺ T cells [58, 424].

In the intestinal immune system it is evident that CD103⁺CD11b⁺ DCs migrate to the MLN during homeostasis and present antigen to CD4⁺ T cells in part inducing an iTreg cell response [24, 35, 323]. However, neither the migration of CD103⁺CD11b⁻ intestinal DCs nor the cross-presenting capacity of intestinal DCs has been assessed, although DCs carrying apoptotic epithelial cells can be detected in rat mesenteric lymph [64]. In addition, it is interesting that CX3CR1^{hi} cells in the lamina propria have high levels of MHCII and co-stimulatory molecule expression yet apparently do not migrate to the MLN during homeostasis [35]. This raises the suggestion that CX3CR1^{hi} cells may play a role in modulating the function effector T cells via re-stimulation in the periphery as has recently been described for Langerhans cells in the skin [100]. It is also possible that CX3CR1^{int} or CX3CR1^{hi} cells acquire some migratory capacity under inflamed conditions as myeloid cell migration in this context has not been assessed. Lastly, both CX3CR1^{int} cells and CX3CR1^{hi} cells have been suggested to have a more inflammatory phenotype responsible for driving inflammation [25, 413, 427]. However, rather conversely we and others have also shown substantial IL-10 production from the CX3CR1^{hi} cell population which is likely to promote tolerance [308, 311, 428]. Therefore, the ability to study specifically the properties and functions of the different myeloid cell subsets described in this thesis is paramount

to extend this work. The specific immediate experiments suggested to address this are outline below.

Immediate future experiments to probe functional characteristics of intestinal myeloid cell subsets

- Elegant work tracking fluorescent antigen uptake by different DC populations over time has indicated the ways in which migratory and lymphoid tissue resident DCs co-operate in inducing immune responses. A similar approach tracking the distribution of orally administered fluorescent ovalbumin over time in the above tolerance or oral vaccine settings (described in section 6.2) in CX3CR1^{GFP/+} mice would enable the co-operation of intestinal CD103⁺ and CX3CR1⁺ cell subsets in antigen uptake and migration to the MLN to be assessed.
- Although the ability to selectively deplete specific myeloid cell subsets while leaving the rest of the network intact is currently limited, our knowledge of the ontogeny of these subsets and the *in vitro* system for DC differentiation described here offers the opportunity to selectively reconstitute mice with distinct subsets following DC depletion (described in section 6.2). For example, such selective reconstitution with monocytes should repopulate only the CX3CR1⁺ cell compartments, whereas conversely reconstitution with *in vitro* derived CD103⁺CD11b⁺ DCs would repopulate only this compartment. Initially, an assessment of the effect of selective reconstitution of DC depleted mice on the inflammation induced by *H. hepaticus* and anti IL-10R treatment would offer a useful proof of concept experiment.

- In preparation for a longer term research strategy the CD11c.iDTR mice (iDTR denotes a ROSA26-FLX-STOP-FLX-DTR allele [492]) were deliberately acquired because they offer the potential to more specifically delete myeloid cell populations by crossing iDTR mice to novel Cre-recombinase expressing strains as they become available. For example, crossing iDTR mice to CX3CR1-Cre mice could be used to generate a CX3CR1.iDTR strain and so deplete CX3CR1⁺ cells.

6.4 The differentiation of DC subsets *in vitro*

The ability to differentiate DC subsets *in vitro*, such as is described in this thesis for CD103⁺CD11b⁺ DCs, offers the potential to manipulate their function for therapeutic value. CD103⁺CD11b⁺ DCs are a major antigen presenting subset which appears to preferentially induce Treg cell responses during homeostasis and so it seems natural to try and utilise this population to prevent intestinal inflammation from developing [157, 158]. Two immediate experiments are suggested to address this.

Immediate future experiments utilising *in vitro* derived CD103⁺ DCs in a therapeutic manner

- *In vitro* differentiated CD103⁺ DCs could be adoptively transferred into mice prior to the induction of colitis by DSS treatment or into RAG^{-/-} mice prior to the induction of colitis by T cell transfer. The relative development of inflammation would then be assessed relative to controls receiving no DCs or DCs differentiated in the presence of GM-CSF or FLT3L alone.
- *In vitro* differentiated CD103⁺ DCs could be loaded with bacterial antigen (e.g. ShelAg derived from *H. hepaticus*) and adoptively transferred into

mice in an attempt to generate antigen specific tolerance to intestinal bacteria. The subsequent effect of the adoptive transfer of DCs upon *H. hepaticus* induced inflammation could then be assessed.

6.5 The role of IL-27 in co-ordinating the intestinal immune response

In this thesis we correlated the expression of IL-27p28 and IL-10 in germ-free, conventionally-housed mice and *H. hepaticus* infected mice. In addition we showed IL-27p28 expression was significantly up-regulated during *H. hepaticus* induced colitis. These findings were discussed in detail in the context of current literature in section 5.3.3, but it is worth highlighting here the important immediate experiments to further these observations.

Immediate future experiments to investigate the dual role of IL-27 in the intestine

- EBI-3^{-/-} mice [488], which lack expression of both IL-35 and IL-27, and IL-27R α ^{-/-} mice [293], whose T cells are non responsive to IL-27, have been imported. Analysis of IL-10 expression in the presence or absence of *H. hepaticus* colonisation either by intracellular cytokine staining (in both EBI-3^{-/-} and IL-27R α ^{-/-} mice) or by adoptive transfer of OT-II.FoxP3^{huCD2/+}.IL-10^{GFP/+} CD4⁺ T cells as described in section 6.2 (into EBI-3^{-/-} mice only) followed by ovalbumin feeding would indicate the importance of IL-27 in the regulation of endogenous IL-10 production. In addition an assessment of the colitis induced by *H. hepaticus* infection with concurrent anti IL-10R treatment in IL-27R α ^{-/-} mice would indicate whether IL-27 signalling has a functional role driving inflammation.

6.6 The control of immune responses by the intestinal microbiota

This thesis joins a body of recent work analysing the role that the intestinal microbiota can play in modulating the immune response and the contribution this might have in inflammatory diseases. In particular the progression of human diseases, including IBD and obesity, has been linked to alterations in the composition of the microbiota with the expansion or reduction of specific families coming to the fore [163]. For example there is a relative increase in *Enterobacteriaceae* species in IBD patients [384] and a relative increase in *Firmicutes* species in obese individuals [383]. Alongside this work, studies in mouse models have begun to show that the presence and composition of the intestinal microbiota can either ameliorate or exacerbate inflammatory disease depending on the context. Significantly, the influence of the microbiota extends beyond the intestine affecting pathology in models of CNS-inflammation, airway-inflammation and type-I-diabetes [373, 374, 376].

Emerging work has begun to define specific bacterial species which are particularly potent modulators of the intestinal immune response. The presence of SFB can enhance Th17 and Th1 responses and exacerbate pathology in IBD and arthritic models [168, 201, 226, 372]. Alternatively, the outgrowth of *Klebsiella pneumoniae* and *Proteus mirabilis* can induce an infectious form of colitis [190] and *H. hepaticus* itself can induce immune-pathology in immune deficient settings [278]. Conversely, infection of mice with the human commensal *Bacteroides fragilis* or the transfer of a constituent polysaccharide (PSA) can offer protection in multiple models of intestinal inflammation [102, 275, 276, 497]. Furthermore, our data and others show that the microbiota can promote regulatory responses including IL-10 production [472, 473]. We also show specifically that the mouse

commensal *H. hepaticus* can act to promote regulatory responses in immune-sufficient mice although it remains to be seen whether this can offer protection from the development of immune-pathology.

Despite this substantial body of work it remains unclear precisely how colonisation with single strains of bacteria, such as *H. hepaticus*, can cause such profound immune responses and ultimately clinical effects. However, drawing on all these studies and our own, two important themes emerge which should direct future research.

1. Indirect effects of microbial colonisation via alterations in microbial ecology within the intestine.
2. Direct effects of microbial colonisation via the sensing of microbes and microbial products by immune cells and the induction of defined immune pathways.

In support of the former it is clear that intestinal bacteria do not act in isolation. For example, neither *Klebsiella pneumoniae* nor *Proteus mirabilis* will induce colitis in germ-free mice although in conventionally-housed mice the effect is dramatic [190]. Similarly, evidence suggests that the inflammatory properties of *Helicobacter hepaticus* are dependent on the presence of other bacteria [474, 476]. Instead, it is hypothesised that colonisation with or outgrowth of certain bacterial species induces a profound reshaping of the intestinal microbiota which underlies the immune effects. In this way it is often difficult to interpret monocolonisation studies which suggest that specific bacteria induce specific immune responses as this may not be the case in the context of a complete microbiota. Developing a better understanding of how specific bacteria alter the

microbial ecology within the intestine, in particular identification of other bacterial species which may be proportionally enhanced or inhibited by their presence, is thus paramount. Due to the complexity of a conventional microbiota it is impossible to model this reliably in conventionally-house mice. Rather the development of limited mouse microbiotas, such as ASF, that consist of a defined number of specified species which collectively fulfil the minimal microbiome required to set up a sustainable microbial community would provide the means to scientifically assess the impact of specific factors on microbial ecology.

Most immunological focus remains on direct effects that intestinal bacteria have on immune cells and the subsequent immune pathways that are induced. To this end we have shown that the IL-10 response to the commensal bacteria and to *H. hepaticus* is independent of MyD88, ruling out all TLRs except TLR3 and TLR4 in this effect and also IL-1 and IL-18. It is quite clear however that other immune responses to bacterial colonisation such as the regulatory response to *Bacteroides fragilis* and the maintenance of epithelial integrity are MyD88-dependent [102, 275, 276, 185]. Therefore it is reasonable to assume that the immune response to the microbiota is not limited to one defined immune pathway. We highlight IL-27 here as a cytokine which correlates with the IL-10 response and it is interesting that IL-27 can be regulated in a TRIF-dependent fashion rather than by MyD88 alone [498]. Furthermore IL-27 can also be induced by IFN- β which is well established to be a TRIF-dependent cytokine [480]. Therefore, the role of TRIF and type-I IFNs in the IL-10 response in the intestine represent key lines of future research.

Immediate future experiments investigating the immune responses induced by the intestinal microbiota and *H. hepaticus* infection

- The effect of *H. hepaticus* mono-colonisation of germ-free mice or co-colonisation with ASF on the intestinal immune response will be assessed. Furthermore, sequencing of 16sDNA sequences of the microbiome following *H. hepaticus* infection will be undertaken to indicate overall changes in microbial ecology.
- The IL-10 response in uninfected or *H. hepaticus* infected TRIF^{-/-} and IFNAR1^{-/-} will be assessed relative to wild-type controls to determine the dependency of IL-10 production on TRIF- and type I IFN-signalling.

6.7 Translation to the human

Ultimately in order to provide therapeutic targets for the treatment of IBD it is important to translate mechanistic research in the mouse across to the human. For this reason we are keen to utilise the findings presented here to develop models to better study the responses of human DCs to intestinal bacteria in the context of differing susceptibility to IBD development. In particular, the study of human DCs is currently dominated by monocyte derived populations. However, it is unclear how these relate to the DCs present under homeostasis, which in the mouse develop from a pre-DC that is yet to be identified in human blood. In this thesis we described a system for differentiating CD103⁺ DCs from mouse bone marrow. Using similar growth factors to differentiate cord blood derived CD34⁺ progenitors or embryonic stem cells into DCs *in vitro* may provide a similar system to generate human CD103⁺ DCs for further study. In particular comparing the responses of these cells to classical monocyte derived DCs in terms of bacterial

stimulation, effector T cell polarisation and migration within 3D-tissue models would be productive. Furthermore the recent development of technology to differentiate DCs from iPS cells offers the potential to derive relevant populations of DCs from patients with known IBD-susceptibility polymorphisms [499]. In this way it would become possible to characterise the impact of a polymorphism on the DC phenotype directly in a human setting.

Immediate future experiments translating this work to human samples

- Isolate CD34⁺ progenitors from cord blood and culture with FLT3L, TPO and SCF to induce FLT3L-derived DCs as described previously [452]. Then treat differentiated cells with combinations of GM-CSF, IL-4 and retinoic acid to try to promote a CD103⁺ DC phenotype in a manner analogous to the mouse system. The phenotype of DCs derived in this way could then be compared to monocyte derived DCs such as those described by *Iliev et al* [398].

Chapter 7

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