Dendritic cell regulation of immunity to *Leishmania donovani*

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Abstract

Dendritic cells lie at the interface of innate and adaptive immunity, critically contributing to the generation of an antigen-specific immune response. Although the mechanisms by which they initiate protective immunity are well characterised, their precise contribution to the subsequent regulation of immunity, particularly in the context of chronic infection, is less fully described.

Leishmania donovani is an intracellular protozoan parasite of mammalian phagocytes, capable of establishing a persistent and life-threatening infection in man. Associated with profound immunopathology and chronic immune suppression, visceral leishmaniasis is endemic to some of the world's most resource-poor regions. There is no vaccine and current therapeutic options are limited by parasite resistance, high toxicity and prohibitive costs. As such, a deeper understanding of the immunological mechanisms underlying this disease is critical for the development of effective novel interventions.

Phenotypic and functional analysis of $CD11c^{hi}$ conventional dendritic cell subsets (cDCs) revealed widespread alterations as a result of chronic infection in the murine spleen. cDCs displayed a limited capacity for costimulatory molecule expression and pro-inflammatory cytokine production *ex vivo*. Instead, the preferential production of the immunoregulatory cytokines IL-10 and IL-27 led to the establishment of an auto-regulatory cytokine cascade, modulating cDC function and limiting their capacity to direct effector T cell polarisation *in vitro*.

Conditional *in vivo* ablation of $CD11c^+$ cells during chronic infection suggested a key role for DCs in the maintenance of pathology and parasite persistence in the spleen, whilst adoptive transfer approaches revealed for the first time that $IL\text{-}10^+IL\text{-}27^+$ cDCs facilitated the expansion of IL-10 producing Th1 cells *in vivo* and significantly contributed to the progression of disease.

Taken together, this study reveals a paradoxical capacity for cDCs to suppress effective immune responses during chronic parasitic infection and highlights novel immunoregulatory mechanisms associated with this neglected tropical disease.

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Author's declaration

All data presented in this thesis is original. With the exception of parasite quantification by flow cytometry carried out by Asher Maroof (**5.7**) and confocal microscopy image acquisition carried out by Jason Mann (**4.8**), all the work presented here was performed by Benjamin Owens.

Chapter 1: Introduction

1.1 Dendritic cells: potent initiators of adaptive immunity

Dendritic cells (DCs) lie at the interface of innate and adaptive immunity. Originally characterised in a series of seminal studies during the 1970's [1-4], this unique leukocyte population is endowed with an enhanced capacity for stimulating proliferation in a mixed leukocyte reaction (MLR) [5, 6] and for generating effector T cell populations *in vitro* [7]. Initially identified visually by their 'dendritic' morphology and indirectly by their functional properties, the Integrin, alpha X (complement component 3 receptor 4 subunit; ITGAX), CD11c is now used as a surrogate marker for DC identification [8].

The central paradigm of DC function places them as the dominant cell population involved in the initiation of an antigen-specific T cell response [9]. Proposed after observations on the function of mouse Langerhans' cells (LCs), a type of dendritic cell first observed in 1898 and formally given the 'dendritic' nomenclature as early as 1948 [10], a series of studies identified sequential alterations in LC function and location which allowed them to induce naïve T cell activation [11-17]. When isolated from tissue, LCs are in an 'immature' state; able to take up antigen but limited in their ability to stimulate T cells [13]. After 48 to 72 hours of *in vitro* culture, LCs undergo a process of 'maturation', allowing them to much more efficiently activate naïve T cells in a MLR [12].

Associated with this maturation process, DCs begin to process and present antigen via both Major Histocompatibility Complex (MHC) class I and II pathways. The majority of antigens presented in the context of MHCI molecules are derived from the degradation of ubiquitinated endogenous proteins by proteasomes. These peptides reach the endoplasmic reticulum (ER) by associating with specialised proteins known as transporters associated with antigen processing (TAP) -1 and TAP-2 that enables them to associate with newly formed MHCI molecules residing in the ER in a complex composed of the chaperone proteins calreticulin, Erp57 and tapasin. Once peptide has bound this complex, the fully folded MHCI molecule is released from the ER and translocated through the golgi apparatus to the cell surface.

In contrast to the bulk of MHCI antigen presentation, peptides presented in the context of MHCII are generated from exogenous proteins degraded in acidified endocytic vesicles. MHCII molecules are held in an inactive state in the ER by an association with the invariant chain (Ii). Upon access of this complex to acidified endosomes, the invariant chain is cleaved, leaving a short peptide fragment (CLIP) bound within the MHCII peptide-binding groove. Finally, the MHCII-like molecule Human Leukocyte Antigen (HLA)-DM induces the release of CLIP and facilitates the association of peptides generated in the endosome with the now vacant peptide-binding groove. The fully formed MHCII:peptide complex then translocates to the cell surface (antigen presentation reviewed in [18]).

Antigen processing during maturation is concurrent with enhanced expression of MHC surface molecules [11, 15, 16], a process also occurring in response to both DC developmental stage [19] and exposure to inflammatory stimuli [20]. Maturation is also

associated with increased expression of the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) [14] (reviewed in [21]). It is the coordinate expression of these molecules alongside antigen in the context of surface MHCII that allows DCs to provide the two signals required for initial activation of naïve $CD4^+$ T cell populations [22]. T cell activation does not usually occur locally, as DCs are capable of migration to local lymph nodes after activation, [17] (reviewed in [23]). This process is dependent on increased expression of the chemokine receptor CCR7 during maturation [24-26], and enables them to traffic via afferent lymphatic vessels to sources of CCL19 and CCL21 in the T cell zone of the regional lymph node [24, 27, 28].

The sequential processes of antigen uptake, processing, maturation, migration and subsequent T cell stimulation in the lymph node underlie the conceptual basis of DC functionality in the immune system. However the existence of multiple DC subsets, as well as complexities in their ontogeny and function, modulates the confidence with which such behaviour can be universally ascribed to cells of this type [29, 30].

1.2 Dendritic cell heterogeneity

DCs are found in a multitude of lymphoid and non-lymphoid tissues and bear many phenotypic and functional specialisations. Initially revealed to be a component of the splenic lymphoid tissue [1], DCs within steady state lymphoid organs actually comprise a discrete group of subsets with distinct patterns of surface protein expression. So called 'classical' or 'conventional' DCs (cDCs), steady state lymphoid organ resident dendritic cells express high levels of CD11c and MHCII and can be further phenotypically divided into $CD8\alpha^+CD4^-$ ($CD8\alpha^+$), $CD8\alpha^-CD4^+$ ($CD4^+$) and $CD8\alpha^-CD4^-$ (double

negative; DN) cDC subsets [31, 32]. Recent advances in multicolour fluorescence-activated cell sorting and novel genetic marking strategies have facilitated the generation of a detailed map describing the development of conventional DCs in the steady state and the impact of inflammation on this process (**Fig 1.1**).

1.3 Steady state conventional dendritic cell development

Hematopoietic stem cells in the bone marrow give rise to common lymphoid [33] and common myeloid [34] restricted progenitors (CLPs and CMPs, respectively), although emerging evidence suggests that such a binary lineage segregation may be an oversimplification [35, 36], particularly in humans [37]. Early adoptive transfer studies suggested that distinct cDC subsets were derived from either CLPs or CMPs, generating nomenclature designating $CD8\alpha^+$ cDCs as 'lymphoid' DCs and $CD8\alpha^-$ cDCs as 'myeloid' DCs [38, 39]. Although cDCs in spleen and thymus can derive from both CLPs and CMPs when these progenitors are transferred into irradiated animals, the majority of splenic cDCs are derived from macrophage-DC progenitors (MDPs) [40-42], thus sharing a developmental origin with cells of the monocyte and macrophage lineage.

MDPs are $Lin^-CX3CR1^+CD11b^-CD115^+CD117^+Flt3^+$ and capable of differentiating into monocytes, macrophages and DCs in response to GM-CSF *in vitro* or after adoptive transfer *in vivo* [43]. A further specialised progenitor derived from the MDP is known as the common DC progenitor (CDP- also designated as 'pro-DCs'); first identified as a $Lin^-CD115^+Flt3^+CD117^{lo}$ population in murine bone marrow with a

capacity to produce cDCs and plasmacytoid DCs (pDCs), but not monocytes or macrophages [44, 45].

Differentiation from CDPs to fully formed DCs does not occur directly, with cells passing through immediate DC precursor stages. $CD11c^{+}MHCII^{-}$ DC precursors (pre-DCs) were first identified in peripheral blood and give rise to cDCs and pDCs after migration to the spleen [46]. Dendritic cell precursors exclusively giving rise to conventional DCs (pre-cDCs) are a heterogeneous population of $CD11c^{+}MHCII^{-}$ $SIRP\alpha^{lo}$ cells present in the spleen, able to be segregated into $CD24^{hi}$ and $CD24^{lo}$ populations which respectively differentiate into $CD8\alpha^{+}$ cDCs and $CD8\alpha^{-}$ cDCs *in vitro* and *in vivo* [47]. The existence of an immediate pre-cDC supported earlier evidence that distinct cDC subsets are not immediate precursors of each other [48], but until recently the exact processes leading to the differentiation of cDCs from pre-cDCs *in vivo* remained poorly defined.

In a landmark study, Liu *et al* described the phenotype and function of pre-cDCs *in vivo* and delineated the relationship of these precursors to other myeloid progenitors, their capacity for integration into the DC network and the control of their differentiation by regulatory T cells and growth factor availability [49]. $CD11c^{+}MHCII^{-}$Signal Regulatory Protein (SIRP)-$\alpha^{int}Flt3^{+}$ pre-cDCs comprise 0.2% of bone marrow, 0.03% of blood, 0.05% of spleen (see also [47]) and 0.03% of lymph nodes [49]. Incorporation of BrdU by pre-cDCs is observed in the bone marrow but not the spleen or blood, indicating that the majority of pre-cDC expansion occurs at the site of haematopoiesis, rather than in the periphery. Pre-cDCs express CD62L and migrate through the bloodstream to lymphoid organs, where they terminally differentiate and integrate into the cDC

network, exhibiting classic DC probing morphology [47, 49]. Although they give rise to CD8α$^+$ and CD8α$^-$ splenic cDCs at an altered ratio compared to that in unmanipulated animals ([49] cf. similarly skewed CD8α$^+$ cDC differentiation in [47]), adoptive transfer of pre-cDCs almost exclusively gives rise to cDCs, with a significantly reduced potential to generate monocytes or lymphoid lineage cells compared to unfractionated bone marrow cells. Adoptive transfer of MDPs into non-irradiated recipients demonstrates that MDPs give rise to both CDPs and pre-cDCs, whereas transfer of CDPs gives rise only to pre-cDCs and mature cDCs [49]; confirming the precursor-product relationship between CDPs and pre-cDCs.

FMS-like tyrosine kinase 3 ligand (Flt3L) is a critical growth factor required for the development of lymphoid resident cDCs *in vivo*. Mice deficient in Flt3L have substantial defects in DC development [50], as Flt3L acts at multiple stages of this process due to conserved expression of Flt3 on several DC progenitor populations [51] including CDPs [44, 45] and immediate cDC precursors [47, 49]. This regulation at the level of progenitor cells in the bone marrow by Flt3L was initially proposed as the major process regulating DC homeostasis [52]. However, the regulation of cDC division in the periphery is now thought to play a key role. This is also dependent upon signalling through Flt3 [53], although lymphotoxin-α1β2 (LTα1β2) may be involved in the homeostatic control of CD8α$^-$ cDC populations [54]. As initial studies assessing splenic cDC turnover did not take into account the potential for mature cDC division [55, 56], the rapid turnover of around 3 days originally proposed for splenic cDCs has now been revised to approximately 5 to 7 days [57].

Combined with constant replenishing of the pre-cDC pool from the bloodstream at an estimated rate of up to 4,300 cells per hour [57], cDC division *in situ* is now thought to be a major mechanism of cDC homeostasis *in vivo*. Extrinsic immune factors are also involved in the regulation of peripheral cDC division and therefore homeostasis, as depletion of $Foxp3^+$ Tregs leads to a dramatic expansion of splenic cDCs *in vivo* [58, 59], mediated by Flt3L-driven proliferation [49]. Such tightly controlled systems of differentiation and homeostasis are prone to any alterations in steady state immune function, and as such infection and resultant inflammation can impact significantly upon DC development.

1.4 Dendritic cell development under inflammatory conditions

Derived from the MDP [43], monocytes are a heterogeneous myeloid cell population which develop in the bone marrow and exhibit CCR2-dependent mobilisation to the bloodstream upon infectious challenge [60]. In inflammatory settings, CX_3CR1^{lo} $Ly6C^{hi}$ monocytes can enter inflamed lymphoid tissue and acquire many of the characteristics of DCs, such as antigen processing and presentation, CD11c expression and the capacity to induce naïve T cell proliferation [47, 61-63]. Similar inflammatory DCs (iDCs) can develop at local sites of inflammation, with iDCs taking up antigen and migrating to regional lymph nodes where they control Th1 responses to cutaneous *Leishmania major* infection [64]. Inflammatory monocytes can also develop into TNFα and iNOS-producing 'Tip-DCs' during experimental infection with *Listeria monocytogenes* [60, 65], Leishmania *major* [66], *Trypanasoma brucei* [67, 68] and *Brucella melitensis* [69]. However, their designation as a population distinct from iDCs is not universally accepted.

Although precise functions and patterns of surface protein expression differ, most monocyte-derived iDCs in inflamed lymphoid tissue express CD11c, MHCII and CD11b and do not express the cDC markers CD4 or CD8; making them phenotypically similar to the lymphoid-resident DN cDC subset. However, monocytes are completely restricted to DC development under inflammatory conditions, as the adoptive transfer of monocytes into steady state mice fails to generate cDC subsets in peripheral lymphoid organs [43, 47, 70]. Additionally, iDCs derived from monocytes express intermediate levels of CD11c, in contrast to the high levels of CD11c expression used to define lymphoid cDC subsets, suggesting that monocytes do not give rise to DC populations exactly equivalent to cDCs. Nevertheless, the potential links between iDCs, Tip-DCs and splenic DN cDCs are not robustly defined, and so some functional and phenotypic crossover is a possibility under inflammatory conditions (**Fig 1.1**).

Huge progress was made in the field of DC biology by making use of GM-CSF-elicited DCs derived from murine bone marrow or human monocytes *in vitro* [71, 72]. However, these do not appear to have an *in vivo* counterpart in the steady state, and may be more related to monocyte-derived iDCs as they are not dependent upon Signal Transducer and Activator of Transcription (STAT)-3 for their differentiation; in contrast to cDCs [73]. More recent *in vitro* methods for DC generation have involved culture of murine bone marrow with recombinant Flt3L, allowing the generation of pDCs and cDC subsets more closely resembling steady state splenic DC populations [74-76]. However there are limitations to this approach, as a lack of CD4 and CD8α expression and universal CD11b expression on the subsets makes them phenotypically distinct from their *in vivo* counterparts [74]. As such, the assessment of physiologically relevant

lymphoid resident cDC function is likely to be restricted to studies using populations of primary cells isolated directly *ex vivo*- despite other caveats introduced by this process.

1.5 Conventional dendritic cells: specific subsets - specific function?

Irrespective of their ontogeny, the existence of multiple cDC subsets strongly suggests that distinct types of cDC have unique and divergent functionality in the immune system. This is paralleled by the existence of multiple classes of T cell, with well-characterised and distinct function. This includes $CD8^+$ cytotoxic T cells equipped for the production of perforin and granzymes and capable of initiating the death of infected host cells during a variety of pathogenic insults [77]. Exhibiting a greater diversity of potential functions, $CD4^+$ 'helper' T cells represent a group of effector cells with shared developmental characteristics but highly divergent roles during immune responses. Distinct $CD4^+$ T cell subsets include Th1 and Th2 cells, producing the 'signature' cytokines IFNγ and IL-4, respectively, the characterisation of which established the paradigm of distinct helper T cell classes [78]. More recently it has become apparent that several other $CD4^+$ T cell subsets exist, including IL-17-producing Th17 cells [79], IL-9-producing Th9 cells [80] and germinal centre-generating T-follicular helper cells [81]. In addition, several types of regulatory T cells exist and will be discussed later in this study.

Initial studies proposed that T cell polarising function was strictly segregated between $CD8\alpha^+$ and $CD8\alpha^-$ cDC subsets, with $CD8\alpha^+$ cDCs yielding Th1 responses and $CD8\alpha^-$ cDCs generating Th2 responses *in vitro* and after transfer *in vivo* [82-85]. This differential capacity for Th1/Th2 priming was associated with a higher potential for IL-

12 production by CD8α$^+$ cDCs during infection [86-88], although CD8α$^-$ cDCs can also produce IL-12 under some conditions [89-92]. As such, the concept of distinct cDC subsets only instructing certain helper T cell classes is now thought to be an oversimplification.

However, CD8α$^+$ and CD8α$^-$ cDC subsets possess differential antigen presentation capacity [93] and as such are thought to be functionally specialised for the activation of either CD8$^+$ or CD4$^+$ T cell responses, respectively. Supporting this concept, CD8α$^+$ cDCs most efficiently cross present exogenous antigen on MHCI [94], in part due to specialised antigen handling capacity [95] and the expression of a distinct suite of receptors that recognise cell-associated [93, 96, 97] and soluble [98] exogenous antigen. Reflective of this, CD8α$^+$ cDCs appear to be the dominant subset driving effective CTL responses to many classes of pathogen *in vivo*, due to both conventional and cross presentation of antigen [99-105], with their critical role underlined by the complete absence of antiviral CTL function in mice specifically lacking this cDC subset [106]. However, CD8α$^+$ cDCs are also capable of generating CD4$^+$ effector T cell populations in response to pathogens [103, 107, 108], suggesting that this subset has the capacity for more generalised T cell activation. Furthermore, despite the dominant role reported for CD8α$^+$ cDCs, the CD8α$^-$ subset is also capable of cross presentation under inflammatory conditions [109, 110] and in some situations only a proportion of CD8α$^+$ cDCs seem capable of optimal cross presentation [111] - indicating the potential for plasticity with regard to the functional specialisation of distinct cDC subsets. Nevertheless CD8α$^-$ cDCs favour CD4$^+$ T cell activation in response to pathogen encounter [112, 113] and generate CD4$^+$ T cell responses when specifically targeted with antigen *in vivo* [93, 114], suggesting that intrinsic differences in antigen receptor

expression and processing capacity imprint at least some level of specialisation upon cDC subsets under certain conditions. The extent to which supposedly subset-restricted function may be compensated for by alternative subsets *in vivo* is currently unclear.

1.6 Dendritic cell regulation of immune responses

As previously described, DCs are critical for the optimal initiation of adaptive immune responses. However, they also play key roles in maintaining steady state peripheral tolerance and in the active regulation of immune responses.

Steady state, resting DCs are predisposed to the induction of tolerance rather than immunity in the periphery [115] by inducing T cell anergy [116] or deletion [117-119]; a result of their continuous presentation of self antigen to autoreactive T cell clones [120]. This process is similar to their role during negative selection in the thymus [121-123] (reviewed in [124-126]). However, not all steady state DCs elicit T cell unresponsiveness [127], some are capable of generating immunity and tolerance simultaneously [128] and peripheral tolerance induction is often dependent upon co-stimulatory molecule expression not always apparent on steady-state DCs [119, 129, 130]. Therefore multiple factors in addition to their resting state are thought to control peripheral tolerance induction by DCs, such as indoleamine 2,3- dioxygenase (IDO)-expression by specific splenic DC subsets [131-133].

Although *in vivo* evidence for distinct subsets of tolerogenic DCs is limited, $CD8\alpha^+$ cDCs appear to have a major role in generating peripheral tolerance to cell associated antigen [134, 135], due in part to their preferential expression of the endocytic receptor

DEC-205 [136, 137]. This enables them to more efficiently endocytose apoptotic cells [138-140] and, when targeted with self antigen *in vivo*, initiate peripheral tolerance [141]. Such 'cross tolerance' is critical for avoiding autoimmunity induced by endogenous self-reactive T cell clones [142], highlighting the importance of this cross-presenting cDC subset. However, $CD8\alpha^+$ cDCs and DEC-205 do not solely induce tolerance, evident in the crucial role for the subset in effector CTL generation ([106] and cf. above) in addition to the enhanced $CD4^+$ and $CD8\alpha^+$ T cell responses generated after targeting antigen to DEC-205^+ cDCs under certain inflammatory conditions *in vivo* [143-146]. As such it is likely that peripheral tolerance induction by DCs is part of an inherently flexible approach to T cell stimulation, based upon complex interactions over and above the level of exclusive tolerogenic function mediated by distinct DC subsets.

The generation of regulatory T cells (Treg) also provides a method by which DCs can mediate peripheral tolerance in the steady state [59, 147-151]. Treg induction can also be achieved by the experimental targeting of DCs *in vivo* [152, 153], although it is in the context of inflammatory responses where the DC-dependent induction of Treg is particularly important. Several subsets of $CD4^+$ regulatory T cell have been defined, including $Foxp3^+$ Treg that are comprised of both naturally arising [154-156] and peripherally induced [157] populations. In addition, $Foxp3^-$ populations such as IL-10 producing Tr1 cells [158, 159], IL-35 producing iTr35 cells [160] and IL-10^+ Th1 cells [161, 162] arise in response to a diverse range of pathogens and inflammatory stimuli.

In contrast to DCs resident in mucosal tissues which are often specialised for the generation of $Foxp3^+$ Treg [163, 164], evidence for defined subsets of splenic cDCs that

are capable of Treg induction during inflammation is more limited. Nevertheless, transfer of splenic $CD8\alpha^+$ cDCs can reduce pathology in a murine model of GVHD by inducing IL-10 producing T cells [165] and splenic $CD4^+$, but not DN, cDCs are involved in regulating myelin-reactive T cell responses during experimental autoimmune encephalomyelitis (EAE) [166].

Although falling outside the phenotypic definition of conventional DCs, IL-10 producing $CD11c^{lo}CD45RB^+$ 'regulatory DCs' [167-169] expand in the spleen during experimental infection with *Plasmodium yoelii* [170, 171] and *Leishmania donovani* [172, 173]; thereby inducing $IL\text{-}10^+$ regulatory T cells and suppressing antigen-specific T cell responses. Similar IL-10 producing DCs also favour the generation of Tr1 cells during *Bordetella pertussis* infection [174, 175] and are generated in response to *Schistosoma mansoni* phosphatidylserine [176], hookworm secretory products [177] and virulence factors derived from *Yersinia pestis* [178]. Despite the clear capacity for the initiation of regulatory responses by some types of DC in response to infection, there is currently a considerable gap in our knowledge concerning the potential immunoregulatory function of conventional DC subsets during infection *in vivo*. However, based upon *in vitro* and *in vivo* studies of a wide range of DC types, the molecular pathways leading to the initiation of regulation by DCs are starting to be revealed.

1.7 Pathways to regulatory DC function

Whether inducing tolerance or immunity, a key property of DCs is their capacity to sense subtle changes in their environment. They do this by expressing a range of pattern

recognition receptors (PRRs) that recognise conserved elements from a multitude of infectious organisms, also known as pathogen-associated molecular patterns (PAMPs). These include Toll-like receptors (TLRs), germline encoded mammalian homologues of the *Drosophila melanogaster* Toll protein [179]. Humans and mice have 10 and 12 functional TLRs, respectively [180], which allow for the innate recognition of diverse microbial stimuli and are critical for host defence [181]. However, emerging evidence suggests that TLRs can also recognise endogenous self ligands in the context of inflammation [182, 183], autoimmunity [184, 185] and cancer [186] and can thus potentiate inappropriate inflammatory responses.

The induction of pro-inflammatory cytokine production downstream of most TLRs requires the adaptor protein MyD88 [187] which, via other adaptors such as TIRAP [188, 189] and IRAKs 1 & 4 [190], associates with TRAF6, leading to the direct [191] and TAK1-mediated [192] modulation of NF-κB and MAP kinase activity: resulting in IκBζ - dependent activation of pro-inflammatory cytokine genes such as IL-6 and IL-12 [193] (reviewed in [181]). This process is tightly regulated, with non-functional adaptor protein variants [194], ubiquitin ligase-mediated degradation of signalling molecules [195], microRNAs [196] and RNase enzymes [197] all controlling levels of TLR activation.

TLR ligation can also result in anti-inflammatory cytokine production, with the preferential production of IL-10 through pathogen-induced TLR signalling first described as an immune avoidance strategy by *Yersinia pestis,* a process mediated through TLR2 [198]. It is now apparent that many pathogens exploit TLR2-induced IL-10 production by APCs as a strategy to establish infection, such as *Borrelia burgdoferi*

[199], *Aspergillus fumigatus* [200], *Candida albicans* [201] and *Mycobacterium tuberculosis* [202], although the precise signalling pathways involved are not completely clear. However, the activation of extracellular signal-related kinase (ERK) appears to be a conserved component in the induction of IL-10 downstream of TLR2 in DCs, which occurs as a result of MyD88 or spleen tyrosine kinase (Syk)-dependent pathways, determined by the nature of the ligand [203]. TLR2-induced IL-10 production may also require c-Fos, as DCs deficient in this transcription factor produce less IL-10 in response to TLR2 ligation [204].

Signalling through Dectin-1, a C-type lectin PRR that recognises fungal β-glucans [205], also induces ERK and IL-10 production in a Syk- dependent process [206]. Interestingly, Dectin-1 and TLR2 can both recognise fungal zymosan, with signalling through both receptors required for efficient cytokine production [207]. Recognition of zymosan by both receptors also induces regulatory DCs capable of mediating IL-10-dependent tolerance *in vivo* [208]. This regulatory effect is governed by TLR2 signalling, as cDC production of IL-10 and retinoic acid in response to zymosan induces $Foxp3^+$ Treg and suppresses autoimmunity; an effect abolished in zymosan-treated TLR2-deficient mice [209]. TLR2-mediated IL-10 production appears to be dependent on signalling from TLR2/6 heterodimers, as this generates $IL\text{-}10^+$ regulatory DCs in response to bacterial antigen, whereas TLR2/1 heterodimers do not [178]. Such potent anti-inflammatory effects reveal the diversity of possible responses initiated by DCs in response to microbial encounter and highlight the importance of TLR2 in mediating DC regulatory function.

Alongside defined TLR-induced pathways, the activation of ERK provides a generalised mechanism by which the production of IL-10 by macrophages and DCs is enhanced. Stimuli as diverse as oxidative stress [210], cannabinoids [211] and CpG DNA [212] have been shown to induce ERK phosphorylation, resulting in suppression of IL-12p40 production and augmented production of IL-10 in APCs. In addition, FcγRI ligation induces IL-10 production in macrophages [213], as a result of ERK phosphorylation and the subsequent remodelling of chromatin around the *il10* promoter, allowing enhanced Sp1 and STAT3 binding and augmented IL-10 production [214]. Signalling through FcγRIII has also been shown to result in IL-10 production by splenic DCs [215], in addition to modulating the phenotype of BMDCs, whereby activation of DCs in the presence of immune complex-mediated FcγR signalling leads to impaired DC IL-12p70 production and diminished *in vivo* Th1 responses after adoptive transfer [216].

ERK-independent pathways can also inhibit IL-12 and enhance IL-10 production in dendritic cells, with two signalling pathways involving mammalian target of rapamycin (mTOR) and glycogen synthase kinase-3 (GSK3) favouring DC regulatory function via IL-10 dependent (mTOR) and independent (GSK3) pathways [217]. Irrespective of the signalling pathways involved, DC production of regulatory cytokines such as IL-10 allows for potent suppressive effects on immune function, often accompanied by the induction of regulatory T cell populations. However, other cytokines may also play regulatory roles, and their production by DCs during infection is only beginning to be addressed.

1.8 IL-27: a pleiotropic cytokine with a critical role in immune regulation

A heterodimeric cytokine formed of the Epstein-Barr virus-induced gene 3 (EBI3) and the IL-12p35-related protein p28, IL-27 was first described in 2002 as a cytokine that induced the proliferation of $CD4^+$ T cells and synergised with IL-12 for the induction of Th1 polarisation [218]. The discovery of IL-27 provided a long sought ligand for the orphan cytokine receptor WSX-1 (TCCR) [219], previously shown to be required for optimal Th1 induction and effective immunity to *Listeria monocytogenes* [220] and *Leishmania major* [221]. The crucial role for IL-27 in Th1 polarisation is in part due to the STAT1 and STAT3-mediated induction of T-bet and IL-12Rβ2 expression downstream of a signalling complex comprising WSX-1 and gp130 [222], allowing optimal sensitivity to IL-12 and maximal IFNγ expression [223-227]. IL-27 is therefore associated with multiple Th1 and IFNγ-mediated immune responses [228-237].

IL-27 has another crucial element to its functionality, originally suggested by studies showing that signalling through WSX-1 was required to prevent $CD4^+$ T cell hyperactivity and excessive pro-inflammatory cytokine production in response to parasitic infection [238, 239]. This is due to the differential effects of IL-27 at distinct stages of $CD4^+$ T cell activation, with IL-27 favouring the initial activation and STAT1-dependent Th1 polarisation of naïve T cells, but limiting pro-inflammatory cytokine production and proliferation later in activation [240], in part by modulating T cell IL-2 production [241, 242]. These seemingly disparate functions have led to studies showing directly opposed roles for IL-27 in models of the same inflammatory disease, such as both the initiation [228, 234] and attenuation [243] of collagen-induced arthritis.

This capacity for regulating Th1 differentiation is also extended to other T cell subsets, with IL-27 potently suppressing Th17 differentiation [244-246] via STAT1- mediated inhibition of the lineage-determining transcription factor RORγt [247-249]. The induction of IL-27 production by IFNβ *in vivo* also inhibits Th17 cell development, preventing EAE and suggesting a basis for the therapeutic effects of IFNβ in multiple sclerosis [250-252]. However, IL-27 does not appear to affect the function of established Th17 cells [253], highlighting the differential effects of this cytokine on T cell responses, determined by the temporal regulation of its availability.

IL-27 also has the capacity to generate IL-10-producing $CD4^+$ T cells *in vitro* [254-257], via signalling pathways dependent on STAT3 [256]. The optimal generation of $CD4^+IL\text{-}10^+$ cells by IL-27 requires the co-ordinate initiation of c-Maf, ICOS and IL-21 expression [258-260]. In addition, emerging evidence suggests that IL-27 may directly alter methylation patterns around the *il10* promoter in $CD4^+$ T cells, thus allowing greater IL-10 expression [261]. IL-27 also favours the production of IL-10 by IFNγ-producing Th1 cells, although this process appears to depend upon an alternative signalling pathway involving STAT1, STAT4 and Notch [262, 263]. IL-27 also exerts effects on $Foxp3^+$ Tregs, although both positive [264] and negative [265, 266] regulation of Foxp3 expression has been reported.

A direct role for DC-derived IL-27 in the generation of $IL\text{-}10^+$ T cells has been described *in vitro*, where production of this cytokine in response to galectin-1, or after oral tolerance induction with model antigen, favours the differentiation of IL-10-producing T cells with potent regulatory capacity [267, 268]. Evidence for IL-27 production by DCs determining the differentiation of $CD4^+$ $IFN\gamma^+IL\text{-}10^+$ T cells in

response to infection *in vivo* is limited, however the accumulation of IL-27p28 in splenic cDCs during chronic *Leishmania donovani* infection [108] suggests a potential role for this cytokine in diseases associated with immune suppression.

1.9 Visceral leishmaniasis: a neglected tropical disease

The leishmaniases encompass a group of four pathological manifestations resulting from the vector-mediated transmission of the obligate intracellular protozoan *Leishmania* [269]. Caused by over 20 different parasite species and transmitted to humans by around 30 species of sandfly [270], leishmaniasis currently threatens 350 million people and is endemic to 88 countries: 72 of which are classified as developing economies (http://www.who.int/leishmaniasis/en/index.html). Inoculation of the infective promastigote stage of the organism to the mammalian host occurs during feeding by female sandflies, with transformation to the replicating, intracellular amastigote stage occurring after the invasion of, or phagocytosis by, mononuclear phagocytes [271]. Disease takes the form of cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL; also described as kala-azar) and the relatively uncommon post-kala-azar dermal leishmaniasis (PKDL) [272]. Whereas CL and MCL are characterised by cutaneous lesions and the destruction of mucus membranes that can result in disfiguring scars, VL leads to systemic parasitisation of internal organs and is invariably fatal if left untreated [272-274].

Caused by *Leishmania donovani* and *Leishmania infantum*, parasite species' belonging to the *Leishmania donovani* complex [275], an estimated 500,000 new cases of VL are diagnosed each year, resulting in around 50,000 annual fatalities [276]. However, these

numbers are thought to be a significant underestimation, with underreporting of disease in endemic areas a major problem [277-279]. VL is a disease of resource poor and poverty afflicted areas, exemplified by a recent study reporting that VL patients in Bihar, India, faced delays of around 40 days from first presentation to initial contact with a fully-qualified physician: the district in question had 4 healthcare facilities with a resident clinician, serving a population of almost 4 million [280]. As the average cost of drug treatment for one episode of VL is around twice the average monthly income in many areas [281], partial treatment regimens and (financially necessitated) non-compliance is widespread, resulting in significant parasite resistance to the first-line therapeutic agent, sodium stibogluconate (Sb^v) [282, 283].

Even if effective against the strain of parasite, pentavalent antimonials such as Sb^v are highly toxic and associated with severe, sometimes life-threatening side effects [284, 285]. Rising resistance and increasing toxicity in endemic areas [286, 287] has led to the substitution of Sb^v for liposomal amphotericin B [288, 289] as the first-line treatment in many cases, with low, single dose treatments deployed in order to keep down otherwise prohibitive costs [290, 291]. However, Sb^v remains the primary drug used for large numbers of newly diagnosed patients, especially in rural areas [280]. The only oral treatment available for VL is miltefosine [292], the use of which is limited due to high costs. The considerably cheaper drug paromomycin is an antibiotic that is active against a broad range of possible co-infections as well as being more effective than Sb^v [293] and equal to amphotericin B [294] in inducing the complete cure of patients in India. However, this drug appears to have limited efficacy in other endemic areas [295]. Combination therapies involving the simultaneous administration of two or more classes of drug are currently in clinical trials, with initial data suggesting reduced treatment times and higher cost effectiveness as the major benefits of this approach

(reviewed by [296]). Nevertheless, drug resistance and toxicity will likely remain a persistent problem. As visceral leishmaniasis is a disease associated with profound immunopathology, an understanding of these pathological processes is essential for the development of novel therapeutic interventions.

1.10 Visceral leishmaniasis and immune-mediated pathology

Sharing many hallmarks of human disease, the majority of knowledge gained regarding the immunopathology of VL has been derived from mouse models of infection (reviewed in [297]). Although the majority of these involve the intravenous inoculation of relatively high numbers of the amastigote stage of the parasite, models more closely mimicking the natural route of infection are in use [298]. A conserved feature of human and murine disease is the massive enlargement of the spleen and liver, associated with parasitisation of tissue macrophages in these organs, in addition to the bone marrow [271, 297]. In the murine model, the spleen and liver display highly divergent, tissue-specific outcomes after infection [299]. The response in the liver is characterised by the IFNγ, TNFα and LTα-dependent formation of tissue granuloma [300-302]. After an initial period of uncontrolled parasite replication, the formation of these highly organised effector structures allow for the efficient killing of parasites within infected cells, critically dependent upon the induction of iNOS by T or NK cell-derived IFNγ and leading to parasite clearance from this organ at around day 28 of infection [303-308].

Granuloma do not form in the spleen or bone marrow, resulting in the persistent parasitisation of tissue macrophages within these organs [309, 310] (reviewed in [308]).

Although not sufficient to clear the infection, some level of host response occurs in the spleen, demonstrated by increased parasite burdens after neutralisation of IL-12 [311]. Chronic infection of the spleen is associated with the destruction of follicular DCs and germinal centres [312], loss of $gp38^{+}$ stromal cells and the homeostatic cytokines CCL19 and CCL21 [313], as well as extensive TNFα-mediated tissue remodelling and the loss of splenic marginal zone macrophages [314]. In the absence of any therapeutic intervention, splenic parasite burden remains high for the life of the animal.

In contrast to a key role for T cells in protection from disease, B cell deficient mice are highly resistant to the development of VL [315], suggesting a suppressive function for these cells, although this appears to be dependent on polyclonal B cell activation and immune complex formation, rather than B cell cytokine production or antigen presentation (Moore *et al* in preparation and [316]). As opposed to experimental cutaneous *L. major* infection, where BALB/c and C57BL/6 mice are considered susceptible and resistant, due to highly polarised Th2 or Th1 responses, respectively (reviewed in [317]), both experimental strains are susceptible to *L. donovani* infection and the $CD4^{+}$ T cell cytokine profile during infection is mixed [318, 319]. However, host protection is dependent upon expression of IFNγ, with $Ifng^{-/-}$ mice developing uncontrolled visceral infection for 8 weeks after inoculation [320]. As discussed later in this study, the immunosuppressive cytokine IL-10 plays a dominant role during murine and human infection (cf. Chapter 3, reviewed in [321]).

1.11 Genetic susceptibility to *Leishmania* infection

The existence of a single gene that governed the susceptibility of certain mouse strains to infection with *L. donovani* was first suggested in 1977 [322]. It is now clear that Solute carrier family 11 member a1 (*Slc11a1*-previously known as *Nramp1* or *Lsh*), a metal ion transporter expressed in late endosomal and lysosomal compartments [323], determines the early control of *L. donovani* and several other intracellular pathogens in macrophages [324, 325]. This occurs via iNOS dependent [326] and independent [327] mechanisms. It is also expressed in DCs and can regulate antigen presentation by these cells [328], as well as affecting the expression of several immune mediators, such as IL-1β and TNFα; thus modulating systemic responses to intracellular infection (reviewed in [329, 330]). Common laboratory strains of mice such as BALB/c and C57BL/6 bear a mutation in *Slc11a1*, are highly permissive to infection and thus widely used in experimental studies of VL. Mutations in the promoter of this gene also confer susceptibility to VL in man [331] (reviewed in [332]).

1.12 *Leishmania*: a 'quiet invader of mononuclear phagocytes' [308]

As an obligate intracellular parasite, the innate response to *Leishmania* is a critical component in the early control of an infection. Although the signals initiating their recruitment are not clear, neutrophils appear to be the first cells that encounter *Leishmania major* after deposition in the skin and are required for optimal infectivity [333]. The next stages in the establishment of disease are more controversial, with macrophage uptake of apoptotic neutrophils containing viable parasites proposed as a mechanism by which infection can be established [334, 335]. However, other cell types at the site of infection can directly interact with parasites [336] and as such the precise

sequence of events leading to the establishment of productive infection in tissue macrophages is not currently known.

Unlike many other pathogens, *Leishmania donovani* has relatively few well-defined PAMPs and as such, evidence for the recognition of the parasite by a distinct PRR is sparse. However, recognition of other *Leishmania* spp by PRRs is more fully described. *Leishmania major* infection of mice or macrophages lacking the adapter protein MyD88 revealed a requirement for signalling through this pathway in the recognition of the parasite, however as MyD88 is a component of the signalling cascade downstream of the majority of TLRs, in addition to the IL-1 receptor [181], these studies provide little information as to the nature of the initiating signal due to interaction with *Leishmania.* Nevertheless, MyD88 appears to play a role in early IL-1α expression [337] and parasite clearance [338], as well as IL-12 production by DCs, efficient Th1 polarisation and the suppression of Th2 dominated responses *in vivo* [339, 340]. MyD88 deficiency also leads to reduced co-stimulatory molecule expression on splenic DCs and impaired production of IL-12p40 by BMDCs after infection with *L. donovani* [341] and *L. braziliensis* [342], indicating that signalling through this pathway is involved in the response to diverse *Leishmania* spp.

TLR4, which recognises bacterial lipopolysaccharide [343-345], has also been implicated in protection from *L. major* infection, with the TLR4-dependent induction of nitric oxide (NO) and inhibition of arginase required to limit parasite replication *in vitro* and resolve cutaneous infection *in vivo* [346, 347]. Neutrophil activation of macrophages for full killing of *L. major* amastigotes has been shown to require TLR4 [348] and a proteoglycolipid from *L. pifanoi* induces TLR4-dependent cytokine

production and parasite killing by macrophages *in vitro* [349]; suggesting the presence of a conserved moiety recognised by TLR4. In addition, TLR9 appears to recognise the DNA of several species of *Leishmania*, including *L. major* [350, 351], *L. mexicana* [352] and *L. infantum* [353]. However, the specific receptor-ligand interactions occurring during parasite recognition by APCs are still unclear.

In addition to the regulation of cytokine production and co-stimulatory molecule expression by *Leishmania* spp (cf. Chapter 3), parasites are capable of fundamentally altering antigen presentation- a key property of dendritic cells and macrophages and essential for the establishment of adaptive immunity. After internalisation by mononuclear phagocytes, the amastigote forms of *Leishmania* exist in a membrane-bound organelle referred to as the parasitophorous vacuole (PV). Although MHCII is able to reach the PV and is thought to be capable of binding peptide there [354], presentation of parasite antigen to $CD4^+$ T cells via MHCII has been shown to be impaired in macrophages infected with *L. major* and *L. amazonensis*, potentially due to defective loading of peptide onto MHCII, rather than inhibition of antigen uptake and processing or limited translocation of MHC to the cell surface [355, 356].

Leishmania donovani has been shown to inhibit expression of MHCI and MHCII-associated genes in infected macrophages [357], with the promastigote form of the parasite directly limiting presentation of exogenous peptides by cleavage of potential antigens via gp63, a parasite-secreted endopeptidase [358]. *L. major* appears to be capable of intracellular antigen sequestration, linked to a profound defect in the capacity for parasite-specific $CD4^+$ T cell activation during the later stages of macrophage infection *in vitro* [359]. Further supporting the concept of an active suppression of

antigen presentation by *Leishmania* spp is evidence for the lifting of *L. mexicana*-induced suppression of antigen presentation after intracellular killing of the parasite [360], in addition to the production of a small peptide that inhibits MHCII expression in human monocytes after *in vitro* infection with *L. donovani* [361]. However, the observed inhibitory effects may also be due to limited access of appropriate parasite antigen to the presentation pathway, rather than active processes of suppression, as sub-cellular location of parasite antigen appears to be a major determinant in defining the likelihood of presentation on the surface of an infected APC [362, 363].

Although infection appears to impair antigen presentation in macrophages, there is intact presentation of functional MHCII-peptide complexes by GM-CSF-elicited BMDCs infected with live *L. mexicana* [364], as well as the effective *in vitro* stimulation of naïve T cells by *L. major* promastigote- infected BMDCs [365] and *L. donovani* infected human myeloid DCs [366]. Relatively few splenic cDCs are infected *in vivo* [341], suggesting that antigen presentation may not be affected in these cells as a whole. However, the impact of systemic infection on the antigen presenting capacity of splenic cDCs *in vitro* or *in vivo* has not been formally addressed.

1.13 Immunochemotherapy and novel therapeutic interventions

In light of the multiple immunological sequelae associated with VL, combining conventional chemotherapy with immune-related interventions has shown great promise: although mostly limited to the experimental treatment of disease. Early studies showed that the effective therapy of chronically infected mice with Sb^v was critically dependent on T cells, with T cell-deficient nude mice undergoing Sb^v treatment

showing no parasite clearance in response to the drug [367]. This is a result of synergy between Sb^v and pro-inflammatory cytokines, with diminished efficacy of the drug observed in TNFα and IFNγ-deficient hosts after treatment [300, 368]. The link between IFNγ and Sb^v function has also resulted in small scale human clinical trials, with combined administration of recombinant human IFNγ and Sb^v being well tolerated and achieving cure of otherwise refractory patients [369, 370] as well as faster remission times [371]. Presumably also linked to the dependence on IFNγ for optimal chemotherapy, recombinant IL-12 has been used as a successful strategy to enhance the efficacy of Sb^v and amphotericin B in murine models [372, 373]. As discussed later in this study, IL-10R blockade in combination with Sb^v results in remarkable improvements in terms of drug dose and treatment length, highlighting the enormous impact of this cytokine on disease [374].

In addition to cytokine-mediated approaches, modulation of T cell co-stimulatory molecules has also been successful in models of disease. This has comprised two often contiguous approaches; the blockade of negative regulators and the activation of positive regulators- with the eventual goal of enhancing T cell activity. Targets for blockade that have improved parasite clearance and/or potentiated drug treatment have included cytotoxic lymphocyte-associated antigen -4 (CTLA-4) [375, 376], CD86 [377] and PD-L1[378]. Successfully targeted positive regulators of T cell activity include CD40 [372], OX40L [375] and glucocorticoid-induced TNF receptor (GITR) [379]. However, none of these approaches have yet reached clinical trials in man.

1.14 CD11c^{hi} cDCs: part of the pathological picture?

Despite huge advances in our knowledge of the immunopathological processes associated with *L. donovani* infection, several key questions concerning the immunoregulatory mechanisms involved remain. This study aimed to address the limited data concerning the role of conventional CD11c^{hi} in the pathogenesis of chronic VL, in addition to further characterising the regulatory T cell response to infection.

The data presented here reveal that the majority of T cell-derived IL-10 during infection is produced by Foxp3^{-}T-bet^{+} IFNγ -producing effector cells. cDC subsets acquire an immunoregulatory cytokine profile during chronic infection, characterised by production of IL-10 and IL-27. This cytokine profile establishes an auto-regulatory cascade within the CD11c^{hi} cDC population, associated with marked suppression of IL-12p70 production as a result of autocrine IL-10 signalling and a concomitant limitation in the capacity for Th1 polarisation by these cells. Furthermore, conditional ablation and adoptive transfer *in vivo* revealed a critical role for CD11c^{hi} cDCs in the generation of IL-10^{+} effector T cells and for the first time indicated the considerable pathological role played by cDCs in the maintenance of chronic disease. This serves to highlight the functional plasticity of CD11c^{hi} cDCs in the context of chronic inflammation and gives a greater insight into the immunopathological mechanisms underlying this neglected tropical disease.

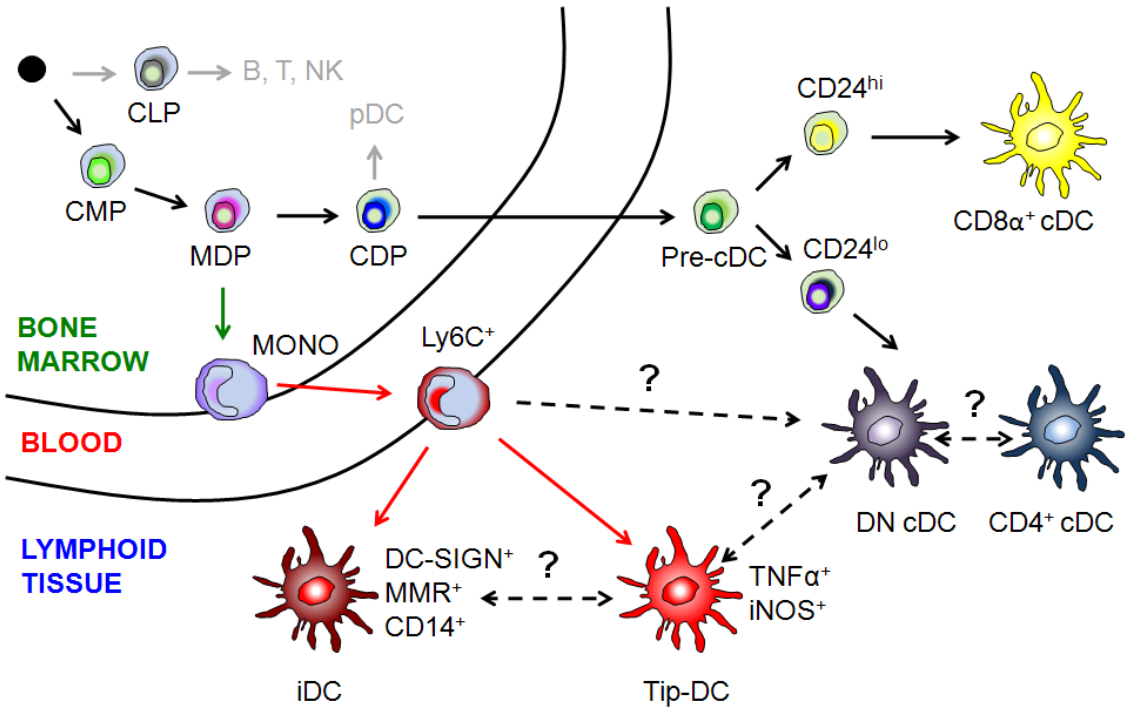

Figure 1.1 Selected stages in the developmental pathway of steady state cDC subsets and inflammatory DCs

Common lymphoid and myeloid progenitors (CLPs & CMPs) develop in the bone marrow from hematopoietic stem cells. CMPs give rise to a common macrophage-DC progenitor (MDP) which develops into monocytes, macrophages and a common dendritic cell progenitor (CDP). This 'pro-DC' gives rise to pDCs, as well as cDCs, via immediate cDC precursors (pre-cDCs). Pre-cDCs traffic through blood to lymphoid tissue where they generate mature cDCs. Distinct cDC subsets arise from distinct pre-cDCs, segregated based on CD24 expression. Under inflammatory conditions, $Ly6C^+$ monocytes can enter inflamed tissue and develop into cells with many DC characteristics, including Tip-DCs and iDCs. The unclear relationship between iDCs, Tip-DCs and $CD8\alpha^-$ cDCs in inflammatory conditions is highlighted. Adapted from [380-382]; original literature cited in text.

Chapter 2: Materials and Methods

2.1 Mice

C57BL/6, B6J.CD45.1 and OTII.*Rag2*$^{-/-}$ mice were obtained from the Biological Services Facility (University of York) or supplied by Charles River Laboratories. B6.*Irf7*$^{-/-}$ mice were originally obtained from the RIKEN BioResource Centre (Ibaraki, Japan) with permission of T. Taniguchi, University of Tokyo. B6.*Il10*$^{-/-}$ mice were kindly provided by Marika Kullberg (University of York).

C57BL/6J-Tg (Itgax-cre,-EGFP) 4097Ach/J (CD11c-cre) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA), stock number 007567 (http://jaxmice.jax.org/strain/007567.html). C57BL/6-*Gt(ROSA)26Sor*$^{tm1(HBEGF)Awai}$/J (ROSA26-STOP-DTR) mice, bearing a simian Diptheria Toxin Receptor under control of the ROSA26 locus with an upstream *loxP*-flanked STOP sequence, were obtained from The Jackson Laboratory, stock number 007900 (http://jaxmice.jax.org/strain/007900.html). Hemizygous CD11c-cre mice were bred with homozygous ROSA26-STOP-DTR mice and genotyped for expression of Cre and eGFP. Genotype positive mice were designated as CD11c.iDTR.

All mice were used between 6 and 12 weeks of age and maintained under specified pathogen free conditions at the University of York. All animal care and experimental procedures were carried out in accordance with United Kingdom Home Office requirements and performed with local ethical approval.

2.2 Infections

Parasites of the Ethiopian strain of *Leishmania donovani* (LV9) were maintained by serial passage through *Rag1/2*$^{-/-}$ mice. Amastigotes were isolated from mechanically dissociated spleens of mice at 3-6 months post infection. Spleen tissue was disrupted in a glass homogeniser containing RPMI-1640 (Sigma, Falkirk, UK). The resulting suspension was centrifuged at 800rpm for 5 minutes and the supernatant retained. Erythrocytes were removed by saponin lysis (Sigma-Aldrich, 0.5mg/ml supernatant) for 5 minutes at room temperature. Parasites were washed three times in RPMI-1640 by centrifugation at 3100rpm for 10 minutes. The resulting pellet was resuspended in RPMI-1640 and amastigotes were counted on a Thoma counting chamber (Hawksley, Sussex, UK).

Mice were infected via the lateral tail vein with $3x10^7$ LV9 amastigotes suspended in 200µl RPMI-1640. Where indicated, acute infection with a high dose ($4.5x10^7$) of parasites was performed. The course of visceral infection was determined by measuring organ mass relative to total body weight (hepatosplenomegaly) and examining methanol fixed, Geimsa-stained (30 minutes stain) imprints of cut sections of the spleen and liver of infected mice. Parasite burdens in organs were quantified as Leishman-Donovan Units (LDU) using the formula: LDU = (number of amastigotes/1000 host cell nuclei) x organ weight (in mg).

Where indicated, parasite load in splenic tissue was determined by flow cytometry. 200µl aliquots of a single cell suspension of splenocytes isolated from infected mice were plated in triplicate in a 96 well v-bottomed plate. Samples were spiked with $5x10^4$

2μM fluorescent microspheres and centrifuged for 5 mins at 3100rpm. Samples were resuspended in 200μl RPMI containing 2mg/ml saponin and centrifuged as before. This was repeated twice. Samples were washed twice in PBS, before being incubated for 20 mins with RPMI containing 2ng/ml CFSE. Samples were washed twice in PBS and fixed in 1% Paraformaldehyde solution, before being acquired on a CyAn flow cytometer (Beckman Coulter).

2.3 Splenic IFNγ production during chronic infection

Naïve mice or mice at days 7, 14, 21, 28 and 56 of infection with *L. donovani* were killed by cervical dislocation. Spleens were isolated and a single cell suspension generated in RPMI-1640 supplemented with 2mM 2-mercaptoethanol (Sigma Aldrich), 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin, (all from Gibco, UK) plus 10% Foetal calf serum (Hyclone, ThermoScientific, USA; Referred to subsequently as Complete RPMI). $10x10^6$ splenocytes were cultured for 72 hours in the presence of 1μg/ml purified hamster anti-mouse CD3ε (500A2, BD Pharmingen, San Diego, CA, USA) or $2x10^6$ fixed *L. donovani* amastigotes (generated in house). Supernatants were harvested and an IFNγ-specific sandwich ELISA (eBioscience, San Diego, California, USA) was performed according to manufacturer's instructions. ELISA plates were washed using an automated SkanWasher plate washer (Molecular Devices, CA, USA) using PBS + 0.05% TWEEN20 (Sigma) as wash buffer. Data was acquired on a VersaMax plate reader (Molecular Devices) and quantified using SoftMax Pro software (Molecular Devices).

2.4 Splenic T cell cytokine and transcription factor expression

Naïve or day 28 infected mice were killed by cervical dislocation. Spleens were isolated and a single cell suspension generated in Complete RPMI. After erythrocyte lysis using Gey's solution, cells were washed twice in RPMI by centrifugation at 1200rpm for 5 minutes and 5-10x10^6 cells were placed in a 96 well plate (Corning Costar). Cells were restimulated for 90-120 minutes at 37^{o}C, 5% CO_2 with 10ng/ml PMA and 1μg/ml Ionomycin before the addition of 1μg/ml Brefeldin A (all from Sigma-Aldrich, UK) for the final 4-4.5 hours of culture.

After restimulation, cells were washed once in RPMI-1640 and twice in Phosphate-buffered saline (PBS) supplemented with 5% FCS and 5% 50mM EDTA (MACS buffer). Cell suspensions were labelled for 30 minutes on ice in 100μl total volume of MACS containing combinations of the following monoclonal antibodies: CD3ε-PE-Cy7 (145-2C11), CD4-FITC (RM4-5), CD127-PE (A7R34), all from eBioscience, CD3ε-PE-Cy7 (Biolegend, San Diego, USA), CD4-PerCP (RM4-5) and CD8α-PerCP (53-6.7, BD Pharmingen). Cells were washed twice in MACS buffer and fixed for 15 minutes on ice in 2% paraformaldehyde (PFA) solution. Where indicated, 1/1000 Fixable Viability Dye eFluor^{780} (eBioscience) was added after fixation. After washing in MACS buffer, cells were permeabilised by washing twice in PBS + 1% Saponin + 1% bovine serum albumin (Sigma) (PERM buffer). Cells were subsequently labelled for 45 minutes on ice in 100μl total volume of PERM buffer containing combinations of the following monoclonal antibodies or appropriate isotype controls: IFNγ-PacificBlue (XMG1.2), IFNγ-eFluor^{450} (XMG1.2), IL-10-APC (JES5-16E3), T-bet AlexaFluor^{647} (ebio4BIO), Foxp3-FITC (FJK-16a) all from eBioscience, IFNγ-APC (XMG1.2), IL-10-PE (JES5-16E3) from BD Pharmingen.

After staining period, cells were washed twice with PERM buffer and once with MACS buffer before resuspending in 200μl MACS buffer and acquiring data on a CyAN-ADP flow cytometer (Beckman Coulter, USA). Subsequent analysis was carried out using Summit^{TM} Software (Beckman Coulter).

2.5 Generation of bone-marrow derived dendritic cells (BMDCs)

Femurs were removed from C57BL/6, B6.*Irf7*$^{-/-}$ or B6J.CD45.1 mice and bone marrow isolated. Cells were washed twice, erythrocytes lysed and resuspended at 1x10^6 cells/ml in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin (all from Gibco, Paisley, UK) plus 10% Foetal calf serum (HyClone) (Referred to subsequently as Complete DMEM). Recombinant murine Granulocyte-Monocyte Colony Stimulating Factor (rmGM-CSF, PeproTech, London, UK) was added to a final concentration of 10ng/ml.

Cells were cultured in T75 tissue culture flasks for 72 hours, when media and non-adherent cells were removed, spun down, resuspended in complete DMEM + 10ng/ml rmGM-CSF and cultured for a further 96 hours. Cultures routinely contained 85-95% CD11c$^+$ cells.

2.6 Antigen-specific T cell restimulation

BMDCs were generated from femurs of C57BL/6 mice as described above. On day 7 of culture, cells were counted and pulsed for 24 hrs with paraformaldehyde-fixed *Leishmania donovani* amastigotes at a ratio of 100 amastigotes to 1 BMDC. Antigen-

pulsed BMDCs were subsequently used to restimulate T cells for 3 hours, prior to addition of Brefeldin A for 4 hours and subsequent assessment of $CD4^+$ T cell cytokine production by intracellular flow cytometric analysis, as previously described.

2.7 Dendritic cell costimulatory molecule expression

Spleens were isolated from naïve and infected mice as previously described. Tissue was dissociated mechanically using a scalpel and digested in 10ml/spleen of RPMI-1640 supplemented with 0.2mg/ml collagenase type IV / DNAse1 mix (Worthington Biochemical, NJ, USA) for 30 minutes at room temperature. 200µl of 50mM EDTA was added to each sample which was subsequently passed through a 100µm cell strainer (BD Bioscience) with MACS buffer to generate a single cell suspension. After erythrocyte lysis with Gey's solution, cells were washed twice and labelled for 30 minutes on ice in 100µl total volume of MACS containing combinations of the following monoclonal antibodies or appropriate isotype controls: CD11c-PE-Cy7 (N418), Major Histocompatibility complex class II (MHCII)-APC (M5/114.15.2), MHCII-eFluor^{450} (M5/114.15.2), CD8α-FITC (53-6.7), CD4-APC (RM4-5), CD40-PE (1C10), CD80-FITC (16-10A1), CD86-APC (GL1), B7-H1-PE (MIH5), TLR2-PE (6C2), CD70-PE (FR70), CD11b-eFluor^{450} (MI/70), CD103-FITC (2E7) all from eBioscience, I-Ad/I-Ed-PE (2G9), Ly-6C-FITC (AL-21) and CD4-PerCP (RM4-5) from BD Pharmingen. After labelling, cells were washed twice in MACS buffer and resuspended in 200µl 2% PFA for subsequent data acquisition and analysis by flow cytometry.

2.8 Dendritic cell subset isolation and sorting

Spleens were isolated from naïve or chronically infected mice and collagenase digested as above. After digestion a single cell suspension was generated, erythrocytes were lysed and cells washed twice in MACS buffer. Dead and highly phagocytic cells were removed by incubating splenocytes for 5 minutes at room temperature with unconjugated basic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by washing and passing the cell suspension over an LS column (Miltenyi Biotec) held in a strong magnetic field (Miltenyi Biotec). The flow-through from the column, depleted of dead and highly phagocytic cells, was washed and incubated with microbeads conjugated to an anti-CD11c (N418) antibody (Miltenyi Biotec) for 30 minutes on ice. After washing in MACS buffer, the cell suspension was passed over an LS column in a magnetic field. $CD11c^+$ DC were retained on the column and eluted and washed in 5ml MACS buffer. The CD11c enriched splenocytes were subsequently labelled for 45 minutes on ice in 500µl total volume of MACS containing the following monoclonal antibodies: CD11c-PE-Cy7 (N418), DX5-PE (DX5), CD4-APC (RM4-5) and CD8α-FITC (53-6.7), all from eBioscience. After staining, cells were washed twice and resuspended in 1.5-2ml of MACS buffer. Cells were sorted to high purity on a MoFlo cell sorter (Beckman Coulter) based upon expression of CD11c and one of either CD4 or CD8α. Double negative (DN) cDCs were sorted based on a lack of CD4 or CD8α expression. Sorted cDC subsets were routinely 98-99% pure.

2.9 cDC subset cytokine profiling by quantitative RT-PCR

cDC subsets were sorted from spleens of individual naïve and chronically infected mice as previously described. Cells were kept on ice throughout sorting and subsequent

washing, resuspended in 350µl RLT lysis buffer (Qiagen, California, USA) containing 1% β-Mercaptoethanol (Sigma-Aldrich) and frozen at -80^{o}C until required.

Lysed cells were defrosted and total RNA was extracted using an RNAeasy spin column kit (Qiagen), according to the manufacturer's instructions. RNA purity was assessed using NANOdrop technology (Thermo Scientific, Wilmington, USA) and frozen until required, or used immediately for cDNA conversion.

cDNA was synthesised using the SuperScript III reverse transcriptase system (Invitrogen). The manufacturer's protocol was slightly modified as the RNA concentration from small numbers of sorted cells was very low. 8µl of RNA was added to 1µl of 50µM oligo(dT)$_{20}$ and 1µl of 10mM dNTP mix to give a total volume of 10µl. After incubation at 65ºC for 5 min, this mixture was placed on ice. A cDNA synthesis master mix containing 10x RT Buffer, 50mM $MgCl_2$, 0.1M DTT, RNaseOUT (40U/µl) and SuperScriptTM III RT (200U/µl) was prepared. 10µl of this cDNA synthesis mix was added to 10µl of the RNA/Oligo(dT)$_{20}$ mixture, mixed gently and centrifuged briefly at 10,000rpm for ~5 sec. This cDNA synthesis reaction mixture was incubated at 50ºC for 50 min. The reaction was terminated by incubating at 85ºC for 5 min and then chilling on ice. Reaction mixtures were centrifuged briefly and 1µl of RNase H was added and incubated for 20 min at 37ºC. Resulting cDNA was stored at -20ºC or used immediately for qRT-PCR

Real-time quantitative PCR was performed with the SYBR green PCR kit in an ABI Prism 7000 sequence detection system (Applied Biosystems) according to the

manufacturer's instructions. Reaction mixtures contained 12.5µl of SYBR Green, 8.5µl of dH2O, 1µl of forward primer (1µM) and 1µl of reverse primer (1µM) and were combined with 2µl target cDNA to a final volume of 25µl in a MicroAmp Optical reaction plate. Reactions were performed under the following conditions: 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of; 95ºC for 15 seconds, 62ºC for 30 sec and 72ºC for 30 sec, followed by 72ºC for 5 min and a final cooling to 4ºC to terminate the reaction. Expression of target genes was normalized to HPRT and expressed as fold change in expression using the change in cycle threshold ($\Delta\Delta$CT) analysis method. The relative expression of the target mRNA in cDC subsets isolated from chronically infected mice was directly compared to the same subset isolated from naïve mice. Primer sequences for target genes are indicated (**2.28**).

2.10 cDC cytokine production determined by ELISA

Total cDC populations or cDC subsets were sorted from spleens of individual naïve and chronically infected mice of various strains, as previously described. After sorting cells were washed, counted and plated in triplicate in complete RPMI at $1x10^6$ cells/ml ($5x10^4$ cDCs per 50µl media per well). Where indicated, LPS (1µg/ml) (Sigma Aldrich), anti-mouse IL-27p28 (10µg/ml), Goat IgG (10µg/ml) (both from R&D Systems) or anti-mouse IL-10R (1.3µg/ml) (Marika Kullberg) were added to the cDC cultures. After 24 hours, supernatants were harvested and stored at -80^{o}C until required. Sandwich ELISAs specific for IL-10 and IL-12p40 (Mabtech, Stockholm, Sweden) and Quantikine ELISA kits specific for IL-27p28 and IL-12p70 (R&D Systems) were subsequently carried out on cDC culture supernatants, according to manufacturer's instructions. ELISA plates were washed using an automated SkanWasher plate washer (Molecular Devices, CA, USA) using PBS + 0.05% TWEEN20 (Sigma) as wash buffer.

Data was acquired on a VersaMax plate reader (Molecular Devices) and quantified using SoftMax Pro software (Molecular Devices)

2.11 Sorting of naïve OTII.*Rag2*$^{-/-}$ T cells

OTII.*Rag2*$^{-/-}$ mice were killed by cervical dislocation and spleens and LN were isolated. A single cell suspension was generated, erythrocytes were lysed and cells resuspended in 200µl-500µl total volume of MACS buffer. Cells were labelled for 30 minutes on ice with the following monoclonal antibodies: CD44-APC (IM7), TCRβ-FITC (H57-597) and CD62L-PE (MEL-14), all from eBioscience. After staining, cells were washed twice and resuspended in 1-2ml MACS buffer. Naïve T cells were sorted on a MoFlo high-speed cell sorter (Beckman Coulter) based on a TCRβ^{+} CD44^{lo} CD62L^{hi} cell population. Sorted cells were routinely 98-99% pure.

2.12 CFSE labelling of naïve T cells

Sorted naïve OTII T cells were washed twice in plain RPMI. Cells were resuspended in 1ml of warmed (37^{o}C) plain RPMI, followed by addition of 9ml CFSE-containing RPMI to a final concentration of 3µM CFSE. Cells were rested at 37^{o}C in a water bath for exactly 5 minutes. After labelling, cells were washed 3 times with cold complete RPMI containing 20% FCS to remove excess CFSE. After washing, cells were resuspended for culture in RPMI containing 10% FCS, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 5µl/ml Gentamycin (Sigma).

2.13 cDC subset priming of OTII.*Rag2*$^{-/-}$ T cells *in vitro*

cDC subsets and naïve OVA-specific CD4^{+} T cells were sorted as described. 5x10^{4} cells of each cDC subset were cultured with 1x10^{5} CFSE labelled naïve T cells for 5 days in the presence of 5nM of the OTII CD4^{+} T cell epitope, OVA$_{(323-339)}$. After 5 days of culture, cells were restimulated with PMA & Ionomycin and cytokine production assessed by intracellular cytokine staining and flow cytometric analysis, as described earlier. Where indicated, LPS (Sigma-Aldrich) was added to co-cultures to a final concentration of 1µg/ml.

2.14 Effects of cytokine supplementation or blockade on cDC subset priming of OTII.*Rag2*$^{-/-}$ T cells *in vitro*

Where indicated, cDC subset and OTII co-cultures were supplemented with the following exogenous cytokines and cytokine-neutralising antibodies at the indicated final concentrations; 3ng/ml recombinant murine IL-12, 20ng/ml recombinant murine IL-27, 10µg/ml goat anti-mouse IL-27p28, 10µg/ml normal goat IgG Isotype control (all from R&D Systems), 1.3 µg/ml anti-mouse IL-10R (a gift from Marika Kullberg). After 5 days, cultures were restimulated and OTII T cells assessed for intracellular cytokine production as previously described.

2.15 *In vitro* polarisation of OTII.*Rag2*$^{-/-}$ T cells by cDCs from C57BL/6 and B6.*Il10*$^{-/-}$ mice

CD11c^{hi}MHCII^{hi} cDCs were sorted from spleens of naïve C57BL/6 and B6.*Il10*$^{-/-}$ mice, using CD11c-PE (N418) and MHCII-APC (M5/114.15.2) monoclonal antibodies, and

the method previously described. Naïve OTII.*Rag*2$^{-/-}$ T cells were sorted and cultured at a 5:1 ratio in complete RPMI with sorted cDCs from both strains. Where indicated, 3ng/ml recombinant murine IL-12, 20ng/ml recombinant murine IL-27 or both were added to co-cultures. After 5 days, T cells were restimulated with PMA, Ionomycin & Brefeldin A and assessed by flow cytometry for CD4^{+} OTII cytokine production, as previously described.

2.16 Assessment of serum cytokine levels by ELISA

Peripheral blood was obtained by intra-thoracic cardiac puncture of CO_2-asphyxiated naïve or infected mice, prior to cervical dislocation. Blood was allowed to clot, on ice, for 2-6 hours, before centrifugation at 13000 rpm for 10 minutes to separate fluid-phase serum and blood clots. Serum was isolated and frozen at -80^{o}C until required. Cytokine levels in serum were determined by ELISA using Quantikine kits specific for IL-10, IL-12p70 and IL-27p28 (R&D Systems).

2.17 IL-27p28 production by cDCs from naïve C57BL/6 and B6.*Irf7*$^{-/-}$ mice *in vitro*.

CD11c^{hi}MHCII^{hi} cDCs were sorted from naïve C57BL/6 and B6.*Irf7*$^{-/-}$ mice as previously described. cDCs were plated at 1x10^{6} cells/ml and cultured for 24 hours in complete DMEM. Where indicated, LPS (Sigma-Aldrich) and IFNα (PBL Interferon Source, Piscataway, USA) were added to give final concentrations of 1μg/ml and 1000U/ml, respectively. After culture, supernatants were assessed by ELISA for the presence of IL-27p28 using a Quantikine kit (R&D Systems).

2.18 Conditional ablation of $CD11c^+$ cells in CD11c.iDTR mice

Naïve or infected CD11c.iDTR mice received 4ng/g Diphtheria toxin from *Corynebacterium diptheriae* (DTx, Sigma-Aldrich) in 200µl PBS, or PBS alone, intraperitoneally at days 21, 23, 25 and 27 of infection. Effects of DTx treatment on spleen cell composition were determined on isolated splenocytes at day 28 by staining for 30 minutes on ice with combinations of the following monoclonal antibodies; CD11c-PE-Cy7, MHCII-eFluor^{450}, CD8α-FITC, CD4-APC, CD45R-AlexaFluor^{647} (RA3-6B2), NK1.1-PE (PK136), CD11b-eFluor^{450}, Gr-1-PE (RB6-8C5), all from eBioscience and CD3ε-PE-Cy7 (17A2, Biolegend) and assessing frequencies of indicated cell types by flow cytometry.

2.19 Staining of spleen cryosections for confocal analysis

Spleen tissue (~5mm^3 blocks) was isolated and embedded in Tissue-Tek OCT mounting medium (Sakura Finetek Europe, Netherlands) in Tissue-Tek Cryomolds (Sakura Finetek) and immediately snap frozen on solid CO_2. Samples were subsequently stored at -80^{o}C until required. 10µm thick sections of frozen tissue were cut on a CRYOSTAT (Leica Microsystems GmbH, Wetzlar, Germany) and adhered to poly-L-lysine coated slides (Thermo Scientific) and air dried before fixation in acetone for 5 minutes at room temperature. Slides were washed in buffer (PBS + 0.05% BSA; PAA Laboratories, Paris, France), before 30 minutes blocking at room temperature with PBS + 0.05% BSA + 5% Goat serum. Sections were delineated with an ImmEdge pen (Vector Laboratories, CA, USA) and blocked with an avidin / biotin block kit (Invitrogen) for 15 mins with each reagent, according to the manufacturer's instructions.

Slides were washed and combinations of the following monoclonal antibodies were added in ~50µl buffer containing 5% goat serum per section; CD169-FITC (3D6.112, AbD Serotec, Kidlington, UK), CD3ε-Biotin (eBio500A2), B220-647 (RA3-6B2), F4/80-AlexaFluor^{647} (BM8), MHCII-Biotin (M5/214.1), all from eBioscience, or appropriate isotype controls. Slides were labelled for 45 minutes at room temperature, washed three times and were labelled with 1/200 Streptavidin-AlexaFluor^{546} in buffer containing 5% goat serum for 30 minutes at room temperature. Slides were washed in PBS and counterstained for 5 minutes at room temperature with DAPI diluted to 1µg/ml in PBS. Slides were washed twice and had ~40µl ProlongGold (Invitrogen) applied to allow mounting of coverslips (ThermoScientific). Slides were allowed to cure overnight at 4^{o}C, protected from light, before edges of coverslips were sealed with nail varnish. Slides were kept at 4^{o}C and protected from light until assessment by confocal microscopy.

2.20 Assessment of nitric oxide production by adherent splenocytes

Splenocytes from naïve and infected PBS or DTx-treated mice were plated in complete RPMI at 5x10^{6} cells/ ml for 60 minutes at 37^{o}C. After incubation, wells were vigorously washed using plain RPMI to remove non-adherent cells. 1ml of complete DMEM was then added to wells and cells cultured for 24 hours at 37^{o}C. After culture, supernatant was frozen and stored at -80^{o}C. Nitric oxide levels in supernatant were determined using a Greiss Reagent System (Promega, Madison, WI, USA), according to manufacturer's instructions.

2.21 *In vivo* neutrophil depletion

Naïve or infected C57BL/6 or infected CD11c.iDTR mice were treated with 250µg 1A8 (anti-Ly6G- BioXcell, USA) or 2A3 (isotype control) antibodies by i.p injection at days 21, 23 and 25 post-infection. Neutrophil depletion was determined by assessing the frequency of cells expressing CD11b, Gr-1 and Ly6C in isolated splenocytes by flow cytometry.

2.22 Adoptive transfer of $CD11c^+$ cells into DC-depleted mice

Infected CD11c.iDTR mice were treated at day 21 post-infection with 4ng/g DTx i.p. Approximately 12 hours later, groups of four mice each received $1.5x10^5$ $CD11c^{hi}MHCII^{hi}$ cells, $6.0x10^5$ $CD11c^{int}MHCII^+$ cells, or a mixture of both, sorted from spleens of day 21-infected, congenic wildtype B6J.CD45.1 mice. Mice were subsequently depleted of endogenous DTx sensitive DCs at days 23, 25 and 27, as previously described. At day 28, mice were killed and disease parameters measured as previously described.

2.23 Phenotypic analysis of B6.*Irf7*$^{-/-}$ mice by flow cytometry

C57BL/6 and B6.*Irf7*$^{-/-}$ mice were killed and spleens isolated. A single cell suspension was generated and red blood cells lysed with Gey's solution. Splenocytes were washed and labelled on ice for 30 minutes with combinations of the following monoclonal antibodies; CD11c-PE-Cy7, MHCII-eFluor^{450}, CD8α-FITC, CD4-APC, TLR2-PE, CD3ε-PE-Cy7, CD4-FITC, CD8α-PerCP, MHCII-eFluor^{450}, CD19-PE, CD11b-eFluor^{450} and Gr-1-PE, all from eBioscience, and assessed by flow cytometry as previously described.

2.24 Activation of splenic cDCs by TLR agonists *in vitro*

$CD11c^{hi}$ dendritic cells were sorted from spleens of C57BL/6, B6J.CD45.1 and B6.*Irf7*$^{-/-}$ mice as described previously. Cells were cultured at $5x10^4$ cells/well in 50 µl complete RPMI for 24 hours. As indicated, DC were stimulated with 1µg/ml LPS, 10µg/ml PAM_3CSK_4 (Invivogen, San Diego, USA), 100µg/ml Poly (I:C) (Sigma-Aldrich, St. Louis, USA) or 10µg/ml ODN:1668 (Invivogen). Where indicated, 1000U/ml IFNα (PBL Interferon Source, Piscataway, USA) was added to the culture at 0hr. In some experiments, *B6.Irf7*$^{-/-}$ splenic cDCs were cultured at a 1:1 ratio with sorted congenic wildtype B6J.CD45.1 splenic cDCs. At indicated time points post-stimulation, cells were labelled with monoclonal antibodies specific for CD11c, MHCII, CD40, CD80 and CD86 and assessed by flow cytometry for changes in surface expression of MHCII, CD40, CD80 and CD86, compared to unstimulated cDCs.

2.25 Generation of C57BL/6 and B6.*Irf7*$^{-/-}$ microchimeric mice

B6J.CD45.1 mice were injected intraperitoneally with Busulfan (Pierre Fabre Pharmaceuticals, France) in 250µl sterile 0.9% saline (Baxter, Norfolk, UK) at a dose of 20mg/kg and allowed to rest for 24 hours. For bone marrow transplantation (BMT), femurs were removed from C57BL/6 or B6.*Irf7*$^{-/-}$ mice and bone marrow flushed out using a 10ml syringe, 23 gauge needle and 5ml complete RPMI. Cells were washed twice, erythrocytes lysed and cells counted. Between $2x10^6$ and $8x10^6$ bone marrow cells from C57BL/6 or B6.*Irf7*$^{-/-}$ mice were transferred via the lateral tail vein to Busulfan-treated mice and allowed to engraft over a period of 7-14 days before use.

2.26 Dendritic cell activation by TLR agonists *in vivo*

C57BL/6, B6.*Irf7*$^{-/-}$, microchimeric C57BL/6 ➔ B6JCD45.1 and microchimeric B6.*Irf7*$^{-/-}$ ➔ B6JCD45.1 mice were injected i.v. with 5μg/mouse LPS (Sigma-Aldrich) or 5μg/mouse PAM_3CSK_4 (Invivogen, San Diego, USA) in 200μl PBS or 200μl PBS alone. After 24 hours, mice were killed, spleens isolated and prepared for cDC staining as previously described. Splenocytes isolated from individual mice were labelled with combinations of the following monoclonal antibodies; CD11c-PE, CD11c-FITC, CD45.1-PE-Cy7, MHCII-eFlour^{450}, CD40-PE, CD86-APC, all from eBioscience and CD80-FITC (BD Pharmingen) and analysed by flow cytometry for changes in costimulatory molecule expression.

2.27 Statistical analysis

Statistical analysis was performed using a student's T test with the exception of Fig 4.12 where a one-way ANOVA was used. In all cases, $p<0.05$ was considered significant.

2.28 Oligonucleotide sequences for qRT-PCR

HPRT

Forward: 5'-GTTGGATACAGGCCAGACTTTGTTG-3'

Reverse: 5'-GATTCAACCTTGCGCTCATCTTAGGC-3'

IL-10

Forward: 5'-AGGGTTACTTGGGTTGCCAA-3'

Reverse: 5'-CACAGGGGAGAAATCGATGA-3'

IL-12(p40)

Forward: 5'- CATCAAGAGCAGTAGCAGTTCC-3'

Reverse: 5'- GAATACTTCTCATAGTCCCTTTGG-3'

IL-27(p28)

Forward: 5'-GGCCATGAGGCTGGATCTC-3'

Reverse: 5'- AACATTTGAATCCTGCAGCCA-3'

IDO

Forward: 5'-CGGACTGAGAGGACACAGGTTAC-3'

Reverse: 5'-ACACATACGCCATGGTGATGTAC-3'

TGFβ

Forward: 5' –GCGTGCTAATGGTGGAAAC-3'

Reverse: 5' –CGGTGACATCAAAAGATAACCAC-3'

2.29 Monoclonal antibodies used for flow cytometry

Manufacturers

eBioscience, San Diego, CA, USA

BD Pharmingen, San Diego, CA, USA

AbD Serotec, Kidlington, Oxon, UK

Biolegend, San Diego, CA, USA

Dendritic cells

CD11c-PE-Cy7 (N418) and CD11c-PE-Cy7 (N418)

CD11c-PE (N418)

CD11c-AlexFluor^{488} (N418)

MHCII-APC (M5/114.15.2)

MHCII-eFluor^{450} (M5/114.15.2)

CD8α-FITC (53-6.7)

CD4-APC (RM4-5)

CD40-PE (1C10)

CD80-FITC (16-10A1)

CD86-APC (GL1)

B7-H1-PE (MIH5)

TLR2-PE (6C2)

CD70-PE (FR70)

CD11b-eFluor^{450} (MI/70)

CD103-FITC (2E7)

CD45.1-PE-Cy7 (A20)

I-A^{d}/I-E^{d}-PE (2G9)

Ly-6C-FITC (AL-21)

T cells

CD3ε-PE-Cy7 (17A2) and 1/400 CD3ε-PE-Cy7 (145-2C11)

CD4-FITC (RM4-5)

CD127-PE (A7R34)

CD4-PerCP (RM4-5)

CD8α-PerCP (53-6.7)

IFNγ-PacificBlue (XMG1.2)

IFNγ-eFluor^{450}(XMG1.2)

IL-10-APC (JES5-16E3)

T-bet AlexaFluor^{647} (ebio4BIO)

Foxp3-FITC (FJK-16a)

IFNγ-APC (XMG1.2)

IL-10-PE (JES5-16E3)

CD44-APC (IM7)

TCR-β-FITC (H57-597)

CD62L-PE (MEL-14)

Other cells

Gr-1-PE (RB6-8C5)

NK1.1-PE (PK136)

CD45R-AlexaFluor^{647} (RA3-6B2)

CD19-PE (1D3)

Tissue staining

CD169-FITC (3D6.112)

CD3ε-Biotin (eBio500A2)

CD45R-AlexaFluor^{647} (RA3-6B2)

F4/80-AlexFluor^{647} (BM8)

MHCII-Biotin (M5/114.15.2)

Chapter 3: Immunoregulatory mechanisms during chronic *Leishmania donovani* infection

3.1 Introduction

Immune suppression has a long association with both experimental and human visceral leishmaniasis. Studies as early as 1985 revealed that T lymphocytes in chronically infected hamster spleen failed to proliferate in response to antigen or T cell mitogens and proposed the existence of a lymphocyte population acting in a suppressive fashion during infection [383]. This was complemented by studies showing the direct suppression of T cell responses by adherent spleen cells from susceptible mouse strains during chronic *Leishmania donovani* infection [384]. Accumulation of *Il10* mRNA in $CD4^+$ T cells from *L. major* infected BALB/c mice gave the first indication that *Leishmania* infection may be associated with this prototypical immunoregulatory cytokine [385], followed by the observation that IL-10 expression correlated with pathology in patients with *L. donovani* infection [386] and was reduced after successful treatment [387] .

Evidence for the central role played by IL-10 in murine *L. donovani* disease progression came from observations that $Il10^{-/-}$ mice were highly resistant to infection [388] and that therapeutic blockade of IL-10 signalling with an IL-10 receptor-specific monoclonal antibody during infection significantly enhanced parasite clearance and allowed for a 'near cure' under some conditions [389]. Recent advances in T cell phenotyping have revealed T cell populations distinct from conventional regulatory T cells as major producers of IL-10 in both murine and human infection with *L. donovani* [390, 391], with murine NK cells [392], $CD11c^{lo}CD45RB^+$ DCs [172], human monocyte/macrophages [393] and *in vitro*- derived dendritic cells [394] also producing

IL-10 due to *L. donovani* infection *in vivo* or *in vitro*. Alongside enhanced IL-10 production, impaired secretion of the Th1-promoting cytokine IL-12 by macrophages and DCs has also been reported as a result of *L. major* and *L. donovani* infection [108, 395], indicating the broad impact of these parasites on immune cell cytokine production. The exact phenotype and origin of the IL-10-producing $CD4^+$ T cells which emerge during *L. donovani* infection remain to be elucidated, and it is currently unclear as to whether conventional $CD11c^{hi}$ DCs also acquire an IL-10 producing phenotype as a result of infection.

Despite being instrumental in shaping the resultant immune response to infection, defining the cytokine milieu in isolation is insufficient to fully describe any potential defects in the initiation of a polarised response to a pathogen. In addition to modulation of antigen presentation (cf. Chapter 1), alterations in costimulatory molecule expression and the spatiotemporal access of APC's to T cells bearing a cognate TCR also impact heavily on the functional outcome of the early adaptive immune response. Infection with several *Leishmania* spp has been shown to modulate these key properties.

Alongside presentation of peptide-MHC complexes to T cells bearing a specific cognate TCR, costimulatory molecule expression plays a crucial role in delivering signals required for the optimal induction of an effector T cell response. Impaired expression of the costimulatory molecules CD40, CD80 and heat-stable protein (CD24, [396]) on macrophages during murine *L. donovani* infection has been reported [397, 398], a phenomenon also seen during infection of human monocytes and macrophages with *L. chagasi in vitro* [399]. Infection of murine DCs with *L. amazonensis* amastigotes induces limited surface expression of CD40 when compared to that induced by its

promastigote form [400], a process recently shown to be mediated by the MAP kinase ERK [401]. CD86 appears to be affected differently by infection, with *L. amazonensis* infected human DCs having reduced expression of CD80 but enhanced expression of CD86 [402]. BMDCs encountering *L. braziliensis* had intact upregulation of CD86, but a limited increase in surface CD80. However, when cells which have efficiently internalised parasites were assessed in isolation, no increase in costimulatory molecule expression was detectable [403]. Enhanced CD86 expression on splenic cDCs as well as CD86 dependent activation of $CD8^+$ T cells has been reported early during *in vivo* infection with *L. donovani* [404, 405], suggesting that infection with *Leishmania* spp does elicit a partial activation of DC under some conditions. However the CD86 - CD28 axis has been suggested to be redundant in the early stages of T cell activation in response to infection [377] as well as CD86 blockade being shown to result in enhanced clearance of *L major* [406]; therefore suggesting a neutral or negative correlation between the expression of this costimulatory molecule and effective T cell activation. Less is known about expression of costimulatory molecules on DC during the chronic stages of infection.

Infection-induced segregation of APC and T cells in lymphoid tissue also provides a mechanism by which the initiation of immune response to *Leishmania* can be inhibited. *Leishmania major* promastigotes inhibited splenic DC motility [407] and the migratory capacity of Langerhans' cells *in vitro,* in a process mediated by secreted phosphoglycans [408]. *L. major* is also capable of reducing the chemokine receptor expression and migratory capacity of BMDCs [409], whilst phosphoglycans of *L. donovani* are known to inhibit detachment of cultured human monocytes *in vitro* [410]. Furthermore, evidence from mice chronically infected with *Leishmania donovani* indicated that TNFα and IL-10-dependent inhibition of CCR7 expression on splenic

cDCs impaired their ability to migrate within lymphoid tissue *in vivo* [313], suggesting that *Leishmania* spp are capable of modulating DC migratory capacity, thus limiting their ability to reach crucial areas of lymphoid tissue and initiate optimal T cell activation.

Despite some variability in the impact of *Leishmania* infection upon of macrophage and DCs, a picture emerges of impaired or at best sub-optimal functionality with regard to cytokine production, costimulatory molecule expression and the correct anatomical localisation of APCs during infection. However the majority of the previous dendritic cell oriented studies have focussed on *in vitro* systems, often utilising BMDCs, or have been restricted to assessing DC function during the early events of acute infection *in vivo*. The studies described in this chapter therefore aimed to extend the phenotypic analysis of IL-10 producing T cells that expand during chronic infection, in addition to addressing the paucity of data regarding the impact of chronic *L. donovani* infection upon the phenotype and function of conventional splenic $CD11c^{hi}$ dendritic cell subsets isolated directly *ex vivo*.

3.2 Results

3.2.1 Progressive experimental visceral leishmaniasis is associated with impaired splenic IFNγ production

Female C57BL/6 mice were infected with *L. donovani* amastigotes via the lateral tail vein and infection allowed to develop over a period of 56 days. Mice developed pronounced splenomegaly from day 21 post infection (p.i.) (**3.1A**), associated with an increasing parasite burden in this tissue, most dramatic between days 21 and 28 p.i. and persisting to the end of the experimental infection at day 56 (**3.1B**). Hepatomegaly was also evident in these mice, although alterations were greatest at days 28 and 56 p.i. (**3.1C**). In contrast to the spleen, parasite burden in liver tissue began to be controlled by day 28 p.i. and by day 56 p.i. had been reduced to levels similar to that observed during the early stages of infection (**3.1D**).

In order to make an assessment of any systemic effects of infection on the ability of spleen cell populations to produce IFNγ, whole splenocytes were stimulated for 72 hours with a CD3ε-specific monoclonal antibody (αCD3) or killed parasites (LV9) as a source of antigen. Splenocytes from naïve mice stimulated with αCD3ε produced 4961 ± 378 pg/ml after 72 hours of culture (**3.1E**). Infection led to only marginally enhanced production of IFNγ at days 7 and 21 p.i. (5014 ± 267 pg/ml and 5490 ± 506 pg/ml, respectively). By day 28 p.i., IFNγ production by splenocytes was significantly lower than that of naïve animals (3625 ± 32pg/ml; $p < 0.01$). IFNγ production by splenocytes from mice at day 56 of infection was similarly reduced (3045 ± 394pg/ml; $p<0.01$).

To determine whether antigen specific IFNγ production was also altered during the course of infection, splenocytes were stimulated with $2x10^6$ paraformaldehyde-fixed *Leishmania donovani* promastigotes. As expected, antigen stimulation of splenocytes isolated from naïve animals for 72 hours did not elicit detectable levels of IFNγ production (**3.1F**). Robust antigen-specific IFNγ production was detected at day 7 and 14 p.i. (7241 ± 446pg/ml and 6245 ± 1040pg/ml respectively). However, IFNγ production was reduced by 50-60% at days 28 and 56 p.i when compared to splenocytes from animals at day 7 p.i. Therefore, infection with *Leishmania donovani* leads to persistent parasitisation of splenic tissue and is associated with impaired antigen-specific production of IFNγ in the spleen during the chronic stages of infection.

3.2.2 IL-10 producing $CD4^+$ T cells expand in number and frequency during chronic *Leishmania donovani* infection

Multiple cell types could contribute to the IFNγ response observed above. Therefore to more precisely determine the ability of T cells to produce IFNγ and other potentially immunoregulatory cytokines, intracellular cytokine staining was performed on splenocytes after restimulation *in vitro*.

Non specific restimulation with PMA and Ionomycin for 2hrs revealed populations of splenic $CD3^+CD4^+$ T cells capable of producing IFNγ alone, IL-10 alone or IFNγ and IL-10 simultaneously in both naïve (upper panel) and day 28 infected (lower panel) mice (**3.2A**). However, infection led to dramatic changes in both the frequency (**3.2B**) and total number (**3.2C**) of all three splenic T cell populations. T cells capable of solely producing IFNγ increased significantly in frequency from 2.4 ± 0.4% in spleens of

naïve mice to 44.4 ± 3.0% ($p<0.001$) in mice by day 28 of infection, mirrored by an increase in the number of $CD4^+$ $IFN\gamma^+IL\text{-}10^-$ cells ($1.5x10^5 \pm 4.8x10^4$ vs $1.2x10^7 \pm 2.2x10^6$ cells in naïve and infected mice, respectively; $p<0.01$). $CD4^+$ T cells producing only IL-10 represented 0.2 ± 0.02% of $CD3\varepsilon^+CD4^+$ splenocytes in naïve animals, increasing in frequency to 0.5 ± 0.05% of this population in spleens from mice at day 28 p.i, equating to a 10 fold increase in total $CD4^+$ $IFN\gamma^-IL\text{-}10^+$ cells per spleen. Most strikingly, infection was accompanied by a 50 fold increase in the frequency (0.1 ± 0.02% vs 5.0 ± 0.9% in naïve and day 28 infected mice, respectively; $p<0.001$) and a ~170 fold expansion in the number of splenic T cells capable of simultaneous production of IFNγ and IL-10 ($7.6 \pm 1.2\ x10^3$ vs $1.3x10^6 \pm 1.7x10^5$ in naïve and day 28 infected mice, respectively; $p<0.001$).

To assess antigen-specific T cell polarisation during infection, *L. donovani*-pulsed BMDCs were used as a source of APCs for restimulation of T cells *ex vivo*. Stimulation by this method revealed populations of *L. donovani*-specific T cells with phenotypes analogous to those determined previously (**3.2D**). Again, the frequency (**3.2E**) and number (**3.2F**) of all three splenic $CD3\varepsilon^+CD4^+$ T cell populations was significantly enhanced due to infection. *L. donovani* specific, IFNγ producing $CD4^+$ T cells increased more than 300-fold in frequency (0.1 ±0.03% to 27.6 ±2.6% in naïve vs day 28 infected mice; $p<0.001$) and their number increased from $2.5\ x10^3 \pm 2.7x10^2$ to $6.8x10^6 \pm 1.1x10^6$ ($p<0.001$). $CD\varepsilon^+CD4^+$ T cells specific for *L. donovani* and capable of IL-10 production alone increased in frequency from 0.2 ±0.04% to 0.5 ±0.11% ($p<0.05$) and in number from $6.6 \pm 2.3x10^3$ to $1.2x10^5 \pm 2.5x10^4$ by day 28 of infection ($p<0.01$).

Mirroring the phenotype observed after PMA and Ionomycin restimulation, a population of splenic $CD3\varepsilon^+CD4^+$ T cells capable of the simultaneous production of IFNγ and IL-10 expanded significantly in number from $4.3x10^2 \pm 1.1x10^2$ in naïve animals to $6.4 \pm 1.5x10^3$ ($p<0.001$) cells in spleens isolated from mice at day 28 of infection, representing an ~1471 fold increase in the number of antigen specific $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells in the spleen as a result of chronic infection with this parasite.

3.2.3 T-bet$^+$ Th1 cells are the predominant IL-10-producing T cell population during chronic infection

In order to further investigate the phenotype and potential origin of the $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells, populations of Th1 ($IFN\gamma^+$) Th1 + IL-10 ($IFN\gamma^+IL\text{-}10^+$), Th0 (no cytokine production) and $IL\text{-}10^+$ cells (**3.3A**) were assessed by flow cytometry for intracellular expression of the Th1-associated transcription factor Tbx21 (T-bet), the regulatory T cell-associated transcription factor forkhead box transcription factor 3 (Foxp3), and surface expression of the IL-7 receptor alpha chain (CD127). $CD3\varepsilon^+CD4^+$ T cells capable of simultaneous production of IFNγ and IL-10 were exclusively T-bet$^+$, CD127$^-$ and Foxp3$^-$ (**3.3B**).

In contrast to the dramatic expansion of IL-10-producing Th1 cells, infection led to a slight, but significant, decrease in the frequency of nTreg, with $Foxp3^+$ cells making up $6.31 \pm 0.49\%$ of splenic $CD3\varepsilon^+CD4^+$ cells in naïve animals and $4.54 \pm 0.38\%$ ($p<0.05$) of the $CD4^+$ T cell compartment in mice at day 28 p.i. (**3.3C**). However, these alterations were small and could be attributable to natural variation in $Foxp3^+$ Treg frequency observed between animals. Analysis of the mean fluorescence intensity of

Foxp3 expression in nTreg (**3.3D**) confirmed that $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells did not express detectable levels of this transcription factor.

3.2.4 Chronic infection alters the composition of the conventional splenic DC compartment

In order to assess the impact of infection upon splenic DC subsets, flow cytometric analysis was performed on splenocytes isolated from naïve C57BL/6 mice or those at day 28 p.i. Conventional DCs (cDCs) were defined as $CD11c^{hi}$ $MHCII^{hi}$ cells (**3.4A**) and could be further segregated into three distinct subsets based upon surface expression of CD4 and CD8α (**3.4B**). When assessed by flow cytometry, the frequencies of $CD4^+CD8\alpha^-$ ($CD4^+$), $CD4^-CD8\alpha^-$ (double negative, DN) and $CD4^-CD8\alpha^+$ ($CD8\alpha^+$) cDC subsets in naïve C57BL/6 mice were found to be consistent with that observed by others (**3.4C**) [31, 56]. However by day 28 p.i., all three subsets were significantly altered in frequency.

Comprising the most abundant cDC subset in steady state spleen at 44.2 ± 3.1% of the total $CD11c^{hi}$ $MHCII^{hi}$ cell population, the frequency of the $CD4^+$ cDC subset decreased significantly to 31.6 ± 1.0% ($p<0.05$) by day 28 of infection. There was also a marked alteration in the frequency of the $CD8\alpha^+$ subset, decreasing from 23.9 ± 1.6 % of total splenic cDCs in naïve mice to 9.4 ± 0.5% in the spleen of mice at day 28 p.i. These alterations in the frequencies of the $CD4^+$ and $CD8\alpha^+$ cDC subsets were compensated for by a more than two-fold increase in the frequency of DN cDCs, reflected by an change from 23.9 ± 1.6% of total cDCs at steady state to 54.5 ± 1.5% of the splenic

cDC population by day 28 of infection. Hence, DN cDCs become the dominant splenic DC population in chronically infected mice.

In order to further characterise the phenotype and potential origin of the splenic cDC subsets, splenocytes were stained for expression of the myeloid-associated markers CD11b and Ly6C as well as the integrin CD103, in conjunction with the cDC subset markers used previously (**3.4D-F**). As expected, the $CD4^+$ and DN subsets were $CD11b^+$, whereas the $CD8\alpha^+$ subset showed no expression (**3.4D**). Only the $CD4^+$ and DN cDC subsets showed slight expression of the myeloid marker Ly6C (**3.4E**). $CD4^+$ and DN cDC subsets also appeared to express some level of CD103, with $CD8\alpha^+$ cDCs showing no expression of this integrin (**3.4F**).

3.2.5 Differential regulation of costimulatory molecule expression by cDC subsets during chronic infection

Expression of costimulatory molecules by DCs represents an integral element of their T cell stimulating capacity. In addition, enhanced expression of particular TLRs can result in altered DC function, particularly with regard to cytokine production. The effect of chronic infection upon the cDC-subset restricted expression of the positive costimulatory molecules CD40, CD80, CD86 and CD70 was determined by flow cytometry alongside expression of the negative costimulatory molecule B7-H1 (Programmed –death ligand 1, PD-L1) and the PRR TLR2 (**3.5A-F**).

Splenic cDC subsets displayed differential alterations in expression of costimulatory molecules when naïve mice were compared to those at day 28 p.i. $CD4^+$ and DN cDCs

had similar levels of CD40 expression by day 28 (**3.5A**), increasing by 2.6 ± 0.1 and 2.8 ± 0.1 fold respectively over cDC subsets from naïve mice. $CD8\alpha^+$ cDCs had more modestly enhanced surface expression of CD40, increasing 1.6 ± 0.1 fold over this subset in naïve spleen; significantly less than the $CD4^+$ and DN cDC subsets (both $p<0.01$). The increase in surface expression of CD80 and CD86 in response to chronic infection was muted compared to that of CD40 (**3.5B-C**). $CD4^+$ cDCs responded to infection with a 2.0 ± 0.1fold increase in surface expression of both CD80 and CD86 by day 28, whereas levels of surface CD80 and CD86 on DN cDCs were only 1.5 ± 0.1 and 1.2 ± 0.02 fold higher, respectively, than on DN cDCs from naïve mice. $CD4^+$ cDCs had a significantly enhanced fold upregulation of CD86 compared to the DN subset ($p<0.01$). Neither CD80 nor CD86 expression was increased on the surface of $CD8\alpha^+$ cDCs by day 28 p.i., represented by fold changes in expression of 0.9 ± 0.1 and 0.8 ± 0.1 respectively, with CD80 expression significantly lower than both $CD4^+$ ($p<0.01$) and DN ($p<0.05$) subsets. CD86 upregulation was also significantly impaired on $CD8\alpha^+$ cDCs when compared to $CD4^+$ ($p<0.01$) and DN ($p<0.01$) subsets. In contrast to the previous costimulatory molecules, CD70 expression was increased on all three cDC subsets, which had broadly similar subset-restricted expression patterns (ie; $CD4^+$>DN>$CD8\alpha^+$); although none of these differences were significant (**3.5D**). $CD4^+$ cDCs had a 6.9 ±1.4 fold increase in CD70 expression, DN cDCs a 5.8 ± 0.9 fold increase and $CD8\alpha^+$ cDCs a 4.5 ±1.3 fold increase as a result of chronic infection.

Analysis of cDC subset-restricted expression of the negative costimulatory molecule B7-H1 revealed differential effects of infection to that observed for those previously assessed (**3.5E**). $CD4^+$ and DN cDCs subsets had modestly increased expression of B7-H1 by day 28 p.i., with levels elevated 1.9 ± 0.3 fold and 2.0 ± 0.3 fold over cDC

subsets from naïve animals. In contrast to the lack of expression seen for CD80 and CD86, $CD8\alpha^+$ cDCs had the largest fold increase in surface expression of B7-H1, with levels elevated 3.0 ± 0.6 fold over this subset in naïve mice; significantly greater than that on the $CD4^+$ cDC subset ($p<0.05$). TLR2 expression was increased on all three cDC subsets as a result of infection, with $CD4^+$, DN and $CD8\alpha^+$ cDCs having 4.4 ±0.10, 6.15 ±0.76 and 2.94 ±0.64 fold increases in surface TLR2 expression, respectively, by day 28 when compared to cDCs isolated from naïve mice. There was a trend toward DN cDCs showing the greatest increase in TLR2 expression, although this change was only significantly greater than that on the $CD8\alpha^+$ subset ($p<0.05$).

3.2.6 Sorting splenic cDC subsets to high purity for downstream analysis

In order to gain an insight into the effects of chronic infection upon cDC functionality, it was necessary to isolate highly purified subsets from the spleen of naïve and chronically infected animals. Cells were sorted as $CD11c^{hi}MHCII^{hi}$ (**3.6A**), $DX5^-$ (**3.6B**) and could be further divided into three subsets based upon CD4 and CD8α expression (**3.6C**). Each of the $CD4^+$ (**3.6D**), $CD8\alpha^+$ (**3.6E**) and DN (**3.6F**) cDC subsets were sorted to high purity (>98%) and used for subsequent downstream analysis.

3.2.7 Altered accumulation of cytokine mRNA in cDC subsets during chronic infection

As the cytokine producing potential of DCs impacts heavily upon the functional polarisation of T cell responses after activation, it was of interest to determine what effects chronic infection had upon the expression of mRNA encoding various activatory and regulatory cytokines and mediators in cDC subsets. qRT-PCR of cDC subsets

sorted from naïve mice and mice at day 28 p.i. revealed differential accumulation of cytokine mRNAs within individual subsets.

Infection led to an accumulation of IL-12p40 mRNA in all three subsets, increasing 6 fold in $CD4^+$ cDCs, 5 fold in DN cDCs and 2 fold in the $CD8\alpha^+$ subset isolated from mice at day 28 p.i. over those isolated from naïve mice (**3.7A**). $CD4^+$ and DN cDC subsets both had significantly greater expression of IL-12p40 mRNA than the $CD8\alpha^+$ cDC subset (both $p<0.01$) Assessing the accumulation of Transforming Growth Factor (TGF) β mRNA in cDCs during infection revealed only modest alterations in expression (**3.7B**). $CD4^+$ cDCs showed a 3 fold increase in expression of TGFβ by day 28 p.i., significantly greater than the DN ($p<0.001$) and $CD8\alpha^+$ ($p<0.01$) subsets which did not express higher levels of TGFβ mRNA when compared to these subsets isolated from naïve mice.

Production of the tryptophan catabolising enzyme Indoleamine 2, 3-dioxygenase has been shown to be a mechanism by which distinct subsets of DC are able to mediate immune regulation via suppression of T cell activity [131-133]. Therefore expression levels of IDO mRNA were compared in cDC subsets isolated from naïve and day 28 infected mice (**3.7C**). Chronic infection did not lead to accumulation of IDO mRNA in any of the three subsets when assessed at day 28 p.i. All three cDC subsets accumulated IL-27p28 mRNA as a consequence of chronic *L. donovani* infection, most marked in the DN subset, although only significantly elevated compared to the $CD4^+$ cDC subset ($p<0.05$) (**3.7D**). This was reflected by 3 fold ($CD4^+$ cDCs), 4 fold ($CD8\alpha^+$ cDCs) and 7 fold (DN cDCs) increases in IL-27p28 mRNA accumulation when cDCs isolated from chronically infected mice were compared to those from naïve animals.

Infection led to a dramatic accumulation of mRNA encoding the regulatory cytokine IL-10 in cDCs, particularly marked in the DN and CD8α$^+$ subsets, which both showed significantly higher accumulation than the CD4$^+$ cDC subset ($p<0.01$ for DN; $p<0.05$ for CD8α$^+$; **3.7E**). CD4$^+$ cDCs showed a 4 fold increase in expression of IL-10 mRNA by day 28 p.i., whilst DN and CD8α$^+$ cDCs showed 34 fold and 24 fold upregulation in IL-10 expression, respectively, when compared to subsets isolated from naïve animals.

3.2.8 Impaired production of IL-12p40 and the acquisition of an immunoregulatory cytokine profile by cDC during chronic infection

Despite being useful in initial assessment of any alterations in cDC cytokine profile, mRNA levels in isolation do not allow the definitive assessment of the ability of cells to produce certain cytokines, in particular due to evidence for the posttranscriptional modification of the *Il10* gene during *L. donovani* infection [392]. Therefore cDC subsets were isolated from naïve and day 28 infected mice, cultured for 24 hours and cytokine-specific ELISAs performed on cDC culture supernatant.

Effects of infection upon cDC subset production of IL-12p40, one subunit of a cytokine required for the efficient generation of IFNγ-producing Th1 cells [411], was assessed firstly in cDCs cultured in the absence of exogenous stimulation to determine the levels of spontaneous release in culture (**3.8A**). All cDC subsets isolated from naïve mice produced significantly higher amounts of IL-12p40 protein than those sorted from spleens of mice at day 28 p.i. This was represented by a reduction in CD4$^+$ cDC production of IL-12p40 of 0.49 ± 0.04ng/ml from naïve mice to 0.15 ± 0.01 ng/ml

($p<0.001$) from this subset isolated from mice at day 28. Infection also reduced the capacity for IL-12p40 production by DN cDCs, with production decreasing from 1.30 ± 0.16 ng/ml in naïve mice to 0.21 ± 0.01 ng/ml ($p<0.01$) by day 28 of infection. Capable of the highest IL-12p40 protein production *ex vivo*, the $CD8\alpha^+$ cDC subset was also similarly impaired in its capacity for IL-12p40 production during infection, reflected by a reduction in IL-12p40 from 2.67 ± 0.32 ng/ml from this subset in naïve animals to 0.24 ± 0.08 ng/ml by day 28 of infection. Therefore, whilst IL-12p40 mRNA levels in splenic cDCs were enhanced as a result of *L. donovani* infection, spontaneous IL-12p40 protein production was significantly reduced.

In order to determine whether infection had altered the ability of cDCs to respond to an unrelated IL-12-inducing stimulus, sorted cDC subsets were cultured for 24 hours in the presence of the TLR4 agonist, Lipopolysaccharide (LPS), before IL-12p40 protein levels in cell culture supernatant were determined by ELISA as before (**3.8B**). Although LPS treatment enhanced IL-12p40 production by all cDC subsets isolated from either naïve or chronically infected mice, total levels of IL-12p40 produced by cDCs was significantly inhibited due to infection with *L. donovani*. $CD4^+$ cDCs from naïve animals produced 2.34 ± 0.08 ng/ml after stimulation with LPS, significantly higher than the 1.30 ± 0.08 ng/ml that $CD4^+$ cDCs isolated from chronically infected mice produced after stimulation. LPS-induced production of IL-12p40 was similarly impaired by infection in DN and $CD8\alpha^+$ cDCs, represented by a reduction in production from 3.38 ± 0.23 ng/ml to 0.83 ± 0.08 ng/ml for DN cDCs and 10.83 ± 0.04 ng/ml to 2.54 ± 0.16 ng/ml for $CD8\alpha^+$ cDCs, when protein production by LPS-stimulated cDC subsets sorted from naïve or day 28 infected mice was measured by ELISA.

As qRT-PCR analysis had indicated that mRNA encoding the immunoregulatory cytokine IL-27 accumulated in cDCs as result of infection (**3.7** and [108]), IL-27p28 was measured in supernatant of cDC subsets sorted from naïve and day 28 infected mice (**3.8C**). No IL-27p28 protein was detectable in the supernatant of cDC subsets sorted from naïve mice and cultured for 24 hours without further stimulation. In contrast, all three cDC subsets produced IL-27p28 protein when isolated from d28-infected mice. $CD8\alpha^+$ and $CD4^+$ cDC subsets produced similar levels of IL-27p28 (321 ± 34 pg/ml vs. 314 ± 51pg/ml, respectively; p=ns), whereas DN cDCs secreted significantly less IL-27p28 (194 ± 18 pg/ml; $p<0.05$ vs. $CD4^+$ cDCs).

In addition to the alterations in IL-12p40 and IL-27p28 producing capacity observed during chronic infection, cDC subsets showed an enhanced propensity for IL-10 production in the absence of exogenous stimulation *ex vivo* (**3.8D**). Infection led to significantly augmented IL-10 production by $CD4^+$ cDCs, increasing from 62 ± 7pg/ml in naïve mice to 618 ± 150 pg/ml ($p<0.05$) by day 28 of infection. $CD8\alpha^+$ cDCs showed a similar response, with levels of IL-10 production increasing from 45 ± 4pg/ml in naïve mice to 1004 ± 231pg/ml ($p<0.05$). IL-10 production by DN cDCs was dramatically enhanced due to infection, with protein levels increasing 20 fold from 112 ± 37pg/ml in naïve mice to 2251 ± 196pg/ml ($p<0.001$) in DN cDCs isolated at day 28 of infection.

As autocrine IL-10 signalling is known to suppress IL-12p40 expression [412, 413], it was of interest to determine whether cDC subsets, which were known to produce IL-10 during infection, were capable of IL-10 uptake during the period of *in vitro* culture. In order to address this, cDCs were sorted from naïve and chronically infected mice and cultured for 24 hours in the presence of a monoclonal antibody specific for the IL-10

receptor (αIL-10R) (**3.8E**). cDC subsets isolated from infected mice produced more IL-10 in the presence of αIL-10R; although this increased production was not at a significantly enhanced level than that observed in the absence of the receptor-blocking antibody. However, cDCs isolated from infected mice again produced significantly more IL-10 than those isolated from naïve animals. Infection led to an increase in IL-10 production by $CD4^+$ cDCs from 56.35 ± 4.74pg/ml to 902.72 ± 226.89pg/ml ($p<0.05$) by day 28 of infection. This was accompanied by highly significant increases in IL-10 protein production from both the DN and $CD8\alpha^+$ cDC subsets, represented by increases from 109.02 ± 1.38pg/ml to 3171.91 ± 170.21pg/ml ($p<0.001$) and from 101.91 ± 45.95pg/ml to 1835.77 ± 174.53 pg/ml ($p<0.001$), respectively, when cDC subsets from naïve mice were compared to those sorted from mice at day 28 p.i.

3.2.9 Effective $CD4^+$ T cell priming and weak Th1 polarisation by cDC subsets isolated during chronic infection

In order to assess whether the alterations in costimulatory molecule expression and cytokine profile of cDC subsets as a result of infection impacted upon T cell activation, splenic cDC subsets were sorted from naïve and day 28-infected mice. cDCs were cultured with carboxyfluorescein succinimidyl ester (CFSE)-labelled sorted naïve $CD44^{lo}$ $CD62L^{hi}$ OTII.R*Rag2*$^{-/-}$ $CD4^+$ T cells at a ratio of 1 cDC : 5 OTII, in the presence of $OVA_{(323-339)}$ peptide. OTII proliferation was then assessed after 5 days of culture by CFSE dilution (**3.9A**).

$CD4^+$ cDCs isolated from chronically infected mice were equally effective at inducing proliferation of naïve OTII T cells as those from naïve animals (90.72% ± 0.24% vs

92.26% ± 0.19% of OTII cells undergoing division, respectively; p=ns, Fig. **3.9B**) $CD8\alpha^+$ cDCs induced similar proliferation levels in OTII, with 90.30 ± 0.64% of OTII having divided in cultures containing cDCs from infected mice compared to 90.07 ± 0.58% (p=n.s) dividing in response to $CD8\alpha^{++}$ cDCs sorted from naïve animals. DN cDCs were also equivalent in their ability to induce OTII proliferation with 89.79 ± 1.30% of OTII dividing in response to DN cDCs from naïve animals and 90.21 ± 0.42% (p=n.s) dividing in cultures containing this subset sorted from chronically infected mice. There were no significant inter-subset differences in the T cell priming potential of cDCs; all subsets were broadly equivalent in their ability to induce naïve $CD4^+$ T cell proliferation *in vitro*.

cDC subsets from both naïve and day 28-infected mice were capable of inducing IFNγ production by OTII T cells, with the majority of IFNγ being produced by T cells having undergone several rounds of division (**3.9A**). However, cDCs from chronically infected mice had only a moderately increased capacity for the polarisation of OTII T cells toward a Th1 response, with $CD4^+$ cDCs having a 1.2 ± 0.07 fold, DN cDCs a 1.05 ± 0.10 fold and $CD8\alpha^+$ cDCs a 1.18 ± 0.03 fold enhanced capacity to generate $IFN\gamma^+$ OTII T cells over the respective subset isolated from naïve mice (**3.8C**). No significant differences in Th1 polarising capacity between the cDC subsets were observed. Despite the production of IL-10 and IL-27 by cDCs and the potential role for these cytokines in the development of IL-10-producing T cells [167, 254] (and discussed later in this chapter), there was no evidence that any cDC subset isolated from chronically infected mice was capable of inducing OTII T cells to produce IL-10 alone, or in combination with IFNγ, after co-culture *in vitro*.

In order to further assess any potential defect in the Th1 polarising capacity of cDCs isolated from chronically infected mice, cDC subsets were sorted from naïve and day 28-infected mice and cultured with sorted naïve OTII T cells as before. Culture media was left plain or contained LPS to a final concentration of 1μg/ml in order to activate cDC cytokine production and subsequent T cell polarisation. The addition of LPS to cDC/OTII co-cultures containing cDCs from naïve mice significantly enhanced the polarisation of OTII toward an IFNγ-producing Th1 phenotype (**3.9D**). This was represented by an increase in $CD4^+$ cDC-induced $IFN\gamma^+$ OTII from 15.64 ± 0.98% to 40.76 ± 0.75% ($p<0.01$) in the presence of LPS, an increase from 13.60 ± 0.76% to 37.47 ± 0.14% ($p<0.01$) when DN cDCs were used to activate OTII in the presence of LPS and from 21.55 ± 0.74% to 34.2 ± 1.41 % ($p<0.05$) of $IFN\gamma^+$ OTII when LPS was added to cultures containing $CD8\alpha^+$ cDCs.

In contrast, cDCs isolated from chronically infected mice showed no enhanced capacity for Th1 induction in the presence of LPS (**3.9E**). cDCs from chronically infected mice showed a moderately enhanced ability to generate Th1 responses, consistent with that seen previously. The addition of LPS had no significant effect on the Th1-inducing capacity of cDCs from chronically infected mice, with percentages of $IFN\gamma^+$ OTII T cells induced by each subset remaining equivalent in the presence or absence of LPS. cDCs from chronically infected mice did not polarise OTII T cells toward an IL-10 producing phenotype in the presence or absence of LPS (not shown). Therefore, in spite of LPS being able to increase levels of IL-12p40 production by cDCs isolated from infected mice, this did not augment Th1 polarisation *in vitro*.

3.2.10 Coordinate inhibition of IL-10 and IL-27 signalling improves the Th1 polarising capacity of cDC subsets isolated from chronically infected mice

As cDCs produced IL-10 and IL-27 during infection and OTII were not polarised toward an IL-10-producing phenotype when cultured with cDCs from infected mice, it was of interest to elucidate the potential impact that these cDC-derived cytokines had on T cell polarisation *in vitro*. To address this, cDC subset / OTII co-cultures were established as previously, with the addition of either control IgG, neutralising antibodies to IL-27p28 (αIL-27p28), IL-10-receptor blocking antibodies (αIL-10R) or a combination of both αIL-27p28 and αIL-10R.

In all cases, the addition of control IgG had little effect on the capacity for Th1 polarisation, a value calculated by dividing the percentage of $IFN\gamma^+$ OTII T cells induced by a cDC subset in cultures containing indicated antibody / antibodies divided by the percentage of $IFN\gamma^+$ OTII T cells induced by a cDC subset in cultures containing media and $OVA_{(323\text{-}339)}$ alone. When assessed in cultures containing cDC subsets isolated from naïve mice, the addition of αIL-27p28 had no impact on the Th1 polarising capacity of any cDC subset. Addition of αIL-10R had no effect on cultures containing $CD4^+$ cDCs (**3.10A**), but augmented the Th1 polarising capacity of DN and $CD8\alpha^+$ cDCs isolated from naïve mice by 3.2 ± 0.2 fold and 2.8 ± 0.3 fold, respectively (**3.10B-C**). This was significantly greater than the addition of control IgG for both the DN ($p<0.01$) and $CD8\alpha^+$ ($p<0.05$) subsets. Adding a combination of αIL-27p28 and αIL-10R similarly augmented the Th1 polarising capacity of DN and $CD8\alpha^+$ cDCs from naïve mice, with dual blockade increasing the capacity to induce Th1 commitment 3.5 ± 0.6 fold and 2.9 ± 0.2 fold, respectively. This enhanced Th1 polarisation was again significantly greater for both DN ($p<0.05$) and $CD8\alpha^+$ ($p<0.01$) cDCs when compared to

the effect of a control IgG, however the addition of αIL-27p28 did not significantly alter the ability for IL-10R blockade alone to improve the Th1 polarising capacity of either cDC subset.

The addition of control IgG had little effect on the capacity of cDCs isolated from chronically infected mice for Th1 polarisation (**3.10D-E**). Blockade of IL-27 signalling in isolation had no effect on the Th1 polarising capacity of $CD4^+$ and DN cDCs isolated from chronically infected mice. In contrast, addition of αIL-27p28 increased the capacity for $CD8\alpha^+$ cDCs to induce Th1 polarisation 2.8 ± 0.1 fold ($p<0.01$; **3.10F**). IL-10R blockade enhanced the Th1 polarising capacity of $CD4^+$ cDCs 3.0 ± 0.8 fold, DN cDCs 4.3 ± 1.3 fold and $CD8\alpha^+$ cDCs 4.6 ± 0.7 fold. Other than for $CD8\alpha^+$ cDCs ($p<0.05$), this increased ability to drive Th1 commitment was not significantly greater than the addition of control IgG alone.

Blockade of IL-10R alongside neutralisation of IL-27p28 improved the capacity for Th1 induction by $CD4^+$ and $CD8\alpha^+$ cDC subsets isolated from chronically infected mice. This was reflected by a 3.8 ± 0.7 fold, 5.5 ± 1.6 fold and 8.0 ± 0.2 fold increase in the ability of $CD4^+$, DN and $CD8\alpha^+$ cDC subsets to polarise OTII toward an IFNγ-producing phenotype, respectively. These enhancements in Th1-generating ability were significantly higher than the addition of control IgG for the $CD4^+$ ($p<0.05$) and $CD8\alpha^+$ ($p<0.01$) cDC subsets. In the case of the $CD8\alpha^+$ cDC subset, αIL-10R in combination with αIL-27p28 led to a significantly enhanced increase in Th1 polarising capacity when compared with αIl-10R alone ($p<0.05$). Although not significantly changed, addition of a combination of αIL-10R and αIL-27p28 to cultures containing $CD4^+$ and

DN cDCs also resulted in a trend toward increased capacity for Th1 induction when compared to IL-10R blockade alone.

3.3 Discussion

The data presented in this chapter demonstrate that alongside a dramatic expansion in the frequency and number of IL-10-producing Th1 cells, there are clear alterations in a number of key mediators of effective splenic DC functionality as a result of chronic infection with *Leishmania donovani*. Infection profoundly alters the composition of the conventional splenic DC compartment, as well as being responsible for differential regulation of costimulatory molecule expression on cDC subsets. cDC subsets show an impaired ability to produce IL-12p40 and instead acquire an IL-10 and IL-27 producing immunoregulatory cytokine phenotype during the chronic stages of infection. Although cDCs are still capable of activating and polarising naïve TCR-transgenic T cells, their capacity for inducing Th1 responses *in vitro* is relatively limited, but can be enhanced by IL-10 receptor blockade and neutralisation of IL-27p28 during priming. Despite production of IL-10 and IL-27, cDCs from chronically infected mice do not stimulate naïve TCR transgenic T cells to become IFNγ^{+}IL-10^{+} co-producing T cells *in vitro*.

Unlike the majority of earlier studies, this investigation focussed on determining the impact of chronic infection upon populations of DCs that are as physiologically relevant as possible. Despite limitations in the process by which DCs are experimentally isolated, assessing the effects of infection with *Leishmania donovani* on DCs sorted to high purity directly *ex vivo* hopefully reduces the caveats introduced when experimental approaches are restricted to *in vitro* infection or the use of bone marrow-derived DCs with poorly-defined *in vivo* counterparts.

Previously shown to be a feature of chronic infection of C57BL/6 mice with *L. donovani* [391, 414], this data confirms that splenic $CD4^+$ T cells acquire an IFNγ and IL-10 co-producing phenotype by day 28 of infection. Furthermore by utilising parasite pulsed BMDC as a source of antigen during restimulation, $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells are revealed as a major component of the antigen-specific T cell response to *L. donovani*, suggesting that cells with this phenotype do arise *in vivo* and are not an artefact of PMA and Ionomycin restimulation. The phenotypic analysis of these cells was extended by characterising them as $Foxp3^-$, T-bet^+ and $CD127^-$, a phenotype of IL-10-producing effector T cell analogous to that found in experimental *Toxoplasma gondii* [162], *Plasmodium yoelii* [415] and *Listeria monocytogenes* [262] infection, in addition to human peripheral blood [416]. Such $Foxp3^-$ $CD4^+$ T cells also appear to be responsible for IL-10 production during cutaneous infection of mice with *L. major* [161, 417], although antigen specific $CD25^+$ $Foxp3^+$ natural regulatory T cells may be playing a more significant role in the establishment of chronic disease in that model [418-420].

IL-10 production by conventional Th1 cells also appears to be a feature of human infections. Expanded numbers of antigen-specific T cells co-expressing IFNγ and IL-10 have been reported in the peripheral blood of patients successfully treated for *L. donovani* infection [421], in patients chronically infected with *Borrelia burgdorferi* [422] and are present in the bronchoalveolar lavage of patients with active pulmonary tuberculosis [423]. Furthermore, splenic $CD4^+$ $Foxp3^-$ T cells accumulate IL-10 mRNA during human *L. donovani* infection [390]. As $Foxp3^+$ regulatory T cells have a great capacity for regulating immune responses [424], it was important to determine whether infection led to an expansion of this population of cells. Infection led to a slight, but significant, decrease in the frequency of $CD4^+$ T cells which express Foxp3, suggesting that these cells are not expanded as a result of *L. donovani* infection. This is consistent

with recent clinical data, indicating that visceral leishmaniasis in humans is not associated with an enhanced number or augmented functionality of natural regulatory T cell populations in spleen or peripheral blood [425]. However, expression of Foxp3 in lesions of patients with an uncommon form of dermal pathology caused by *L. donovani* has been reported [426], but as this is a form of cutaneous infection, it is potentially more similar to the response to *L. major* infection. The data presented here therefore supports the growing body of evidence suggesting that co-production of IFNγ and IL-10 by a population of T-bet^{+} Foxp3^{-} CD4^{+} T cells is a common outcome of chronic infection with this parasite in mouse and man.

Chronic infection led to significant alterations in the balance of cDC subsets in the spleen. In particular the frequencies of the CD4^{+} and CD8α^{+} subsets were markedly reduced. It is unclear whether such alterations in subset ratio are due to a loss of surface CD4 and CD8α expression as a result of their functional activation or due to the death of individual cDC subsets, such as the loss of CD8α^{+} cDCs during bacterial sepsis [427, 428] experimental murine malaria [112] and salmonella infection [429]. Similar alterations in splenic cDC subset ratios are also seen after intravenous administration of LPS [56] and so it is possible that the observed effects are due to non-specific effects of inflammation, rather than an effect of *Leishmania* infection *per se*.

The observed increase in DN cDC frequency could also be explained by an influx of monocyte-derived dendritic cells, known to occur at the site of infection with *L. major* [64] or be representative of an increased number of TNF/iNOS-producing DC ('Tip-DC'), previously shown to play a role in protection from *Listeria monocytogenes* [430]. Although DN cDCs expressed similar levels of CD11b to the cell types mentioned,

further phenotypic analysis revealed them to have low expression of Ly6C; a key marker used for the identification of monocyte-derived DC [64]. In addition, both the types of iDC detailed above show only intermediate levels of CD11c expression, whereas the DN cDCs characterised in this study were derived exclusively from the $CD11c^{hi}$ splenic cDC population. Therefore, it is unlikely that the increased frequency of DN cDCs observed during infection with *Leishmania donovani* represents an influx of monocyte-derived or Tip-DC, but whether these cells are capable of increasing expression of CD11c and can thus phenotypically resemble DN cDCs remains to be determined.

Dendritic cell-restricted expression of the alpha-E integrin CD103 has a well-defined role in the initiation of tolerance and regulatory T cell development. CD103-expressing mucosal DCs in the small intestine have the capacity to induce $Foxp3^+$ Treg [163, 164], as well a subset of $CD8\alpha^+CD103^+CD207^+$ splenic DC, located in the marginal zone, being responsible for tolerance to cell-associated antigens [135]. Despite these previous observations, no expression of CD103 was detectable on $CD8\alpha^-$ cDCs in either steady state or during infection, and only a low level was present on $CD4^+$ and DN cDC subsets.

As previously stated, enhanced expression of costimulatory molecules by APCs is an essential component of their T cell activating capacity. Although numerous studies have identified altered costimulatory molecule expression in macrophages and DCs infected with *Leishmania* spp *in vitro,* studies of costimulatory molecule expression during *L. donovani* infection *in vivo* have mostly been restricted to the acute (5hr p.i.) stages of

infection [108, 404]. This data suggests that differential regulation of costimulatory molecule expression on cDC subsets occurs as a result of chronic infection.

Chronic infection led to increased CD40 expression, most markedly on the $CD4^+$ and DN cDC subsets. However, there are conflicting reports as to the requirement for this costimulatory molecule in the generation of protective immunity during *Leishmania* infection. Early studies indicated that accumulation of IL-12p40, the generation of a Th1 response and macrophage leishmaniacidal activity was significantly impaired in CD40-deficient mice, resulting in an enhanced susceptibility to infection with *L. major* [431]. In addition, infection of *cd40l*$^{-/-}$ mice revealed a crucial role for CD40L-induced IL-12 production by APCs in limiting infection with this parasite [432], suggesting that the CD40 – CD40L axis was critical for control of intracellular parasitic infection. This is supported by a recent study in which murine BMDC, engineered to express high levels of CD40, act as an effective therapeutic when adoptively transferred to infected mice [433]. However, other studies have suggested the initiation and maintenance of a robust Th1 response to *L. major* to be independent of CD40 – CD40L costimulation [434, 435], and instead to be reliant on TRANCE-RANK signalling [436]. Therefore, despite enhanced expression of CD40 on cDCs during chronic infection, it is possible that signalling via this costimulatory molecule may be redundant in respect to the generation of a Th1 response during infection, and is not necessarily indicative of fully effective DC activation.

Expression of CD80 and CD86 on cDC subsets was muted relative to CD40 and broadly similar to that seen at 5hr and 24hr p.i.[108, 404]. The greatest fold increases in expression occurred on the $CD4^+$ subset. This may reflect differential levels of infection between the cDC subsets, although direct infection of DC *in vivo*, at least at the acute

stages of infection, is reportedly absent [341] or very low [405], with no apparent bias in cDC subset infection [108]. No studies have addressed levels of direct cDC subset infection by *Leishmania* in the latter stages of infection, so it cannot be ruled out that the differential expression of costimulatory molecules is due to different levels of cDC subset infection.

As CD70 has been reported to be a key mediator of IL-12-independent Th1 polarisation [437], it was interesting that the expression of this costimulatory molecule was dramatically enhanced on all cDC subsets. This may underlie the capacity for intact cDC subset-induced Th1 polarisation despite the relatively limited production of IL-12p40 protein production by these cells when isolated during chronic infection. However, enhanced CD70 expression may not always be beneficial to the host, as the CD70-mediated depletion of B cells has been reported to result in the destruction of the marginal zone in [438]; pathology similar to that seen during chronic infection with *L. donovani*. As such it cannot be assumed that the enhanced CD70 expression identified in this study would have a positive impact on the progression of disease.

In contrast to the relatively modest increases in expression of CD80 and CD86 on cDCs, chronic infection led to enhanced expression of the negative costimulatory molecule Programmed Death Ligand 1 (PD-L1, B7-H1). Increased expression of this molecule on DC has recently been proposed as a mechanism by which the functional exhaustion of $CD8^+$ T cells is mediated during chronic *L. donovani* infection [378]. Enhanced PD-L1 expression was previously assessed on the whole $CD11c^+$ population, whereas this study revealed distinct subset-specific patterns of expression during infection. In particular, the $CD8\alpha^+$ cDC subset showed the greatest increase in surface PD-L1

expression, whereas the $CD4^+$ cDC subset showed the most modest increase. As $CD8\alpha^+$ cDCs have a well described, dominant role in cross presentation and the generation of effector $CD8^+$ T cell responses to exogenous antigen [94], it is possible that the enhanced expression of PD-L1 observed on this subset would have the most marked effect on the process of $CD8^+$ T cell exhaustion as proposed by Joshi *et al*.

Inhibition of T cell priming by enhanced expression of PD-L1 on DC during viral infection has been proposed to 'overwhelm' any positive effects of costimulatory molecule expression by these cells [439], suggesting that even with the relatively intact expression of costimulatory molecules, particularly CD40, on some cDC subsets during chronic *L. donovani* infection, enhanced PD-L1 expression may abrogate any positive impact of this. Furthermore, 'reverse signalling' via PD-L1 or PD-L2 on DC has been shown to result in their impaired functional maturation [440], providing a potential explanation as to the greater impairment of CD40, CD80 and CD86 expression on the $CD8\alpha^+$ cDC subset, associated in part with their more significantly enhanced PD-L1 expression.

Sorting cDC subsets to high purity from spleens of naïve and chronically infected mice and assessing the accumulation of mRNA encoding several cytokines and immunoregulatory mediators by qRT-PCR yielded interesting results. All cDC subsets accumulated mRNA encoding IL-27, but this was particularly evident in the DN subset. This differs slightly from previous observations in day 28-infected BALB/c mice which had the highest accumulation (5-10 fold) of IL-27p28 mRNA in the $CD8\alpha^+$ cDC subset, only a 2-5 fold increase in DN cDCs and less than 2 fold upregulation in $CD4^+$ cDCs [108]. Nonetheless, the data presented here are broadly supportive of the previous

observations and suggest that IL-27 expression is associated with chronic *L. donovani* infection in both strains of mice. This study extended the cytokine profiling carried out previously to include IL-10. The effects of infection upon IL-10 mRNA were dramatic. Transcripts were 30-40 fold higher in DN and $CD8\alpha^+$ cDCs from infected mice, indicating a vast alteration in transcriptional activity within these subsets, similar to that seen in NK cells as a result of *L. donovani* infection [392].

Despite a clear propensity for cDCs to accumulate mRNA encoding the regulatory cytokines IL-10 and IL-27p28 by day 28 of infection, there were no major increases in expression of other immunoregulatory cytokines and mediators, at least at the mRNA level. There was no alteration in the levels of IDO mRNA in sorted cDCs, indicating that this mechanism of limiting T cell function may not be a component of disease progression. However, previous studies reporting IDO-producing DCs as negative regulators of immunity have focussed on very rare $B220^+$ [133] or $CD19^+$ [131] splenic DCs, and so they may not have been included in the stringent cDC gates used for sorting in this study.

qRT-PCR analysis also revealed only modest accumulation of TGFβ mRNA in splenic cDCs, restricted to the $CD4^+$ subset. This is in contrast to the association of this regulatory cytokine with human *L. donovani* infection [441] and data reporting production of TGFβ by T cells and macrophages of mice chronically infected with *Leishmania infantum* [442-444]; a parasite species' distinct from *L. donovani* also capable of inducing visceralising infection. Although T cell or macrophage production of TGFβ was not directly addressed in this study, previous work has shown no increase in TGFβ production by liver mononuclear or spleen cells as a result of chronic infection

with *L. donovani* [375]. As such the lack of significant TGFβ mRNA accumulation by splenic cDCs observed in this study is perhaps unsurprising. Collectively it would appear that the strain of Leishmania responsible for causing VL determines whether the immunoregulatory cytokine milieu is dominated by both TGFβ and IL-10 or solely IL-10. The reason for slightly enhanced accumulation of TGFβ mRNA in the $CD4^+$ subset remains to be formally addressed, however as this subset is known to also accumulate higher levels of IL-23p19 during infection of BALB/c mice [108], it is possible that this subset could be capable of Th17 induction. Such IL-17 producing cells are known to be associated with protection from *L. donovani* infection in humans [445] however, no evidence for IL-17 production by $CD4^+$ T cells as a result of infection was found during this study.

An important finding of this study was the impaired capacity for IL-12p40 protein production by cDCs during the chronic stages of infection in C57BL/6 mice. This occurred despite observed increases in IL-12p40 mRNA in cDCs as a result of infection, suggesting perhaps that post-transcriptional suppression of IL-12p40 may be occurring (cf Chapter 4). All three cDC subsets sorted from chronically infected mice showed significantly reduced production of IL-12p40 protein when compared to those from naïve animals after 24 hours culture *ex vivo*, as well as an impaired ability for cDCs to produce this cytokine in response to LPS stimulation. For cDCs from both naïve and chronically infected mice, in the absence or presence of LPS, the $CD8\alpha^+$ subset produced the highest amount of IL-12, consistent with observations from other studies [88, 92]. The impairment in IL-12p40 production supplements a large body of data concerning the regulation of IL-12 production by DCs and *Leishmania* infection, with this study extending observations to include $CD11c^{hi}$ splenic cDCs isolated from

infected mice, rather than *in vitro* derived macrophages or BMDCs which comprised the bulk of the cells used for previous studies.

Several *Leishmania* species have been reported to directly suppress IL-12 production by macrophages *in vitro*, including transcriptional inhibition of IL-12 promoter activity by *L. major, L. donovani* and *L. chagasi* [446] and the direct modulation of NF-κB signalling and subsequent IL-12 production by cysteine peptidases secreted by *L. Mexicana* amastigotes [447]. *Leishmania*-derived lipophosphoglycan is also capable of directly suppressing LPS-induced IL-12 production in macrophages, in part due to activation of ERK [448]. Sustained inhibition of IL-12 production is also seen in macrophages after phagocytosis of *L. mexicana* amastigotes [449]. The situation with DCs appears to be more complex. *Leishmania major* infection leads to impaired IL-12 production by BMDC *in vitro* [395], an effect potentially mediated by lipophosphoglycans produced by the parasite [450]. However, Langerhans cells and other types of skin DCs are capable of internalising *L. major* amastigotes, producing IL-12 and activating T cells *in vitro* and *in vivo* [365, 451, 452]. The ability of human DCs to produce IL-12 in response to *Leishmania* infection appears to be strain specific, with *L. major* inducing intact IL-12p70 production in response to CD40 engagement, in contrast to *L. donovani* and *L. tropicana* which are unable to prime DCs for production of this cytokine [453].

Despite an evident capacity for some species of *Leishmania* to suppress production of IL-12 by DCs and macrophages, acute *in vivo* infection with *L. donovani* results in production of IL-12p40 by DC, peaking at 24hr p.i. but returning to baseline levels within three days [454], as well as initiating the rapid mobilisation of pre-formed stores

of IL-12p70 from DC and macrophages within minutes of pathogen encounter [455]. IL-12p40 production by $CD8\alpha^+$ DC has also been reported as early as 5hr p.i with *L. donovani* [108], mediated by a process involving vascular cell adhesion molecule-1 (VCAM-1) and its ligand, very late antigen-4 (VLA-4) [456].

The production of IL-12p70 protein by cDC subsets isolated from BALB/c mice chronically infected with *L. donovani* has been shown to be completely suppressed, due to a lack of accumulation of IL-12p40, rather than IL-12p35, mRNA in these cells [108]. This is in contrast to earlier (5hr) stages of infection, where intact accumulation of both IL-12 p40 and p35 mRNA accompanies production of IL-12p70 protein by cDC subsets [108]. As such, the reduced capacity for IL-12p40 protein production described in this study supports a model by which impaired IL-12 production by DC is acquired as disease progresses. Furthermore, this defect, acquired in the chronic stages of infection, is not restricted to an inability for cDCs to produce IL-12 in response to inflammatory stimuli encountered during infection, as cDCs from chronically infected mice stimulated with a high dose of LPS *ex vivo* also show a significantly impaired capacity for production of IL-12; suggesting a more generally impaired capacity for production of this cytokine.

Defective production of IL-12 by professional APCs is likely to play a significant role in the immunopathology associated with *Leishmania donovani* infection. This is underlined by the fact that infection of *Il12*$^{-/-}$ mice with this parasite leads to exacerbated disease, although this is accompanied by reduced hepatic pathology [457]. Furthermore, abrogating IL-12 function by administration of a neutralising antibody during infection leads to reduced IFNγ production and significantly increased parasite

burdens in spleen and liver at day 28 p.i. [458], although a similar experimental approach suggested that neutralisation of IL-12 only affects liver parasite burden and granuloma assembly in the first 28 days, with effects upon splenic parasite burden restricted to the later time points of infection [311]. Treatment with recombinant IL-12 also indicates the importance of this cytokine for disease progression, with substantially reduced parasite burdens being achieved after therapeutic administration of recombinant cytokine as a result of IFNγ-dependent and independent mechanisms [320, 459]. Therapeutic DC vaccination against *L. major* and *L. donovani* infection both require IL-12, as infusion of IL-12-deficient DCs during these experimental approaches severely impairs their efficacy [391, 460].

In summary, the evidence for a lack of IL-12 production by cDCs during chronic infection and the impaired capacity for LPS-induced secretion of IL-12 presented in this study is concordant with previous observations that IL-12p40 production by DC is reduced to baseline levels 72 hours after infection of C57BL/6 mice [454], that IL-12p70 production by cDC subsets is completely suppressed during chronic infection of BALB/c mice [108] and that *Leishmania*-derived LPG and cysteine peptidases impair IL-12 production and interfere with signal transduction after LPS stimulation of macrophages [447, 448, 461]. In addition, PBMC isolated from patients with active, chronic VL are incapable of IL-12p40 production in response to stimulation with *Leishmania* antigen; a defect that is lifted after drug treatment and eventual cure [462]. Taken together, this suggests that an impaired capacity for IL-12 production may be associated with chronic disease in both murine and human systems.

In addition to an impaired ability to produce IL-12 during infection, cDCs acquired an enhanced capacity for production of the immunoregulatory cytokine IL-10 and the effector / regulatory cytokine IL-27. The regulation of IL-27 production is discussed in further detail in Chapter Four of this study, but the capacity for cDCs to produce IL-10 during chronic infection revealed a potentially intriguing situation in which cDCs, instead of potentiating T cell responses, are actually endowed with a paradoxical capacity for the negative regulation of on-going immune responses.

Concordant with the alterations in cytokine production by cDCs seen in this study, a coordinate suppression of IL-12 production and enhancement of IL-10 production has been reported in *L. major*-infected macrophages, where modulation of CD40 signalling results in impaired p38MAPK function, limited production of IL-12 and a shift toward IL-10 production [463, 464]. This is suggested to be due to the formation of an atypical TRAF6 and Syk-containing CD40 signalosome that promotes IL-10 production and is induced by depletion of cholesterol in the cell membrane [465]. Such dysregulated signalling downstream of CD40 suggests that the enhanced expression of this costimulatory molecule on cDCs during chronic infection observed in this study may actually augment the production of IL-10, similar to that seen during the generation of human tolerogenic DCs *in vitro* [466], rather than the usual situation of enhancing IL-12 production by DCs and macrophages. Further work will be required to determine whether a similar signalosome is also formed in DCs and whether *L. donovani* initiates alterations in DC membrane composition and host signalling analogous to that in *L. major*- infected macrophages.

As mentioned in Chapter 1, activation of ERK provides a mechanism by which IL-10 production is initiated in DCs in response to a diverse range of pathogens. However, there are conflicting reports as to whether the activation of ERK occurs in response to infection or encounter with *Leishmania* spp. As previously mentioned, *Leishmania* LPGs are known to be capable of subverting macrophage production of IL-12; a process which is dependent upon the direct stimulation of ERK by these parasite-derived molecules [448]. Further evidence for ERK activation as a result of *Leishmania* infection comes from studies and that infection of macrophages [467] and DCs [401] with *L. amazonensis* leads to phosphorylation of this MAP kinase. Furthermore, *L. donovani* has been shown to suppress p38MAPK activity and enhance ERK1/2 phosphorylation in human peripheral blood monocytes and a THP-1 monocyte cell line in a contact-dependent manner, leading to suppressed TLR-induced IL-12p40 induction and enhanced production of IL-10 [393].

Despite the activation of ERK reported by the above studies, enhanced ceramide synthesis in *L. donovani*-infected macrophages has been shown to result in the dephosphorylation of ERK1/2 [468, 469] and *L. donovani* promastigotes appear to evade activation of ERK upon infection of naïve macrophages [470], suggesting that ERK activation may not occur in all cell types or upon infection with all stages of the parasite lifecycle. Furthermore, *L. donovani* amastigotes do not express LPGs [471], suggesting that LPG-mediated induction of ERK would play a limited role in generating the DC cytokine profile described in this study.

As previously discussed, PRR signalling strongly influences the cytokine-producing capacity of DCs and macrophages, with TLR2 engagement leading to enhanced ERK phosphorylation [204, 472] and IL-10 production by APCs [473], as well as the direct

transition from IL-12 to IL-10 production by macrophages as a result of TLR2-dependent ERK activation due to stimulation with bacterial teichoic acids [474]. In response to TLR2 signalling in splenic DCs, production of IL-10 and retinoic acid leads to autocrine signalling and enhanced suppressor of cytokine signalling (SOCS)-3 expression and the suppression of pro-inflammatory cytokine production, including IL-12p40, IL-12p70, TNFα and IL-6 [209], suggesting that a potential consequence of enhanced retinoic acid production may be to shift cDCs away from a pro-inflammatory cytokine profile, in addition to its role in the generation of $Foxp3^+$ Treg [163, 164]. However, it is not clear whether *L. donovani* infection results in enhanced retinoic acid metabolism by cDCs at this point.

In addition to TLR2 signalling, engagement of the Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN, CD209) also leads to enhanced ERK1/2 phosphorylation and increased production of IL-10 by DCs [475], as well as the negative regulation of DC costimulatory molecule expression and a switch to IL-10 production due to recognition of *Mycobacterium tuberculosis* [476]. Furthermore, as DC-SIGN has been reported to directly bind amastigotes of *L. pifanoi* [477], *L. mexicana* [478] and *L. infantum* [479], there is the possibility that direct recognition of *Leishmania* by this lectin may be responsible for the observed modulation in cytokine profile. However, functional comparison of the mouse DC-SIGN homologue CIRE showed no capacity for engagement of this receptor by *L. mexicana* [480], suggesting that this may not be the case in the murine system.

Unlike other infectious organisms with well characterised moieties on their surface capable of recognition by TLRs, *Leishmania* spp do not have as clear-cut capabilities

for TLR activation. Perhaps of greatest importance in the context of the altered cDC cytokine-producing capacity reported in this study is the recognition by TLR2 of several species' of *Leishmania,* including *L. donovani*. In agreement with the enhanced expression of TLR2 on cDC subsets from chronically infected mice observed in this study, lipophosphoglycan from *L. major* induces TLR2 expression and IFNγ gamma production by human NK cells [481], in addition to LPG from *L. donovani* promastigotes inducing TLR2-dependent production of NO and TNFα in IFNγ-primed murine macrophages [482]. Whilst these studies reveal components of *Leishmania* capable of signalling through TLR2 and inducing an anti-parasitic response, *L. major* LPG is also able to induce expression of IL-10 [483], SOCS-1 and SOCS-3 [338]; components of a suppressive immune environment and permissive to parasite expansion. Furthermore, TLR2-deficient mice, infected with *L. braziliensis*, show greater IL-12p40 production, enhanced IFNγ expression and reduced disease severity [342], as well enhanced parasite clearance at the site of infection after *L. amazonensis* infection [484]. Although IL-10 expression was not addressed in these *in vivo* studies, the enhanced parasite clearance in both models may suggest that TLR2 signalling has a negative effect on productive immunity to these pathogens. As TLR2 directly recognises components of *L. infantum* [485] and TLR2 signalling is directly modulated by *L. donovani* to favour IL-10 production [393], it is possible that engagement of TLR2 contributes to the switch from IL-12 to IL-10 production and subsequent impairment of immune responses during visceral leishmaniasis.

Despite some ambiguity as to whether ERK is always activated in APCs as a result of interaction with *Leishmania* spp and the currently unclear nature of the PRR engaged by *L. donovani*, ERK-dependent modulation of cytokine responses in DCs after infection allows the generation of a hypothetical model which may go some way toward

explaining the impaired IL-12p40 production and enhanced IL-10 expression by cDCs observed in this study (**3.11**). However, as many of the previous studies were based upon direct infection of APCs *in vitro,* further studies directly assessing the state of ERK activation in defined *ex vivo* cDC populations during chronic infection with *L. donovani* would be required to more fully support this proposed mechanism.

Co-culture of sorted cDC subsets and naïve OTII TCR transgenic T cells allowed an assessment to be made of the priming and polarising capacity of cDCs during chronic infection. All cDC subsets were capable of priming naïve T cells, with those isolated from chronically infected mice no better at inducing proliferation of CFSE-labelled T cells than those from naïve animals. This was despite the fact that they had enhanced expression of some costimulatory molecules. The fact that cDCs were not more impaired in their capacity to generate T cell proliferation may be influenced by the fact that cultures contained the peptide epitope of OVA recognised by the OTII TCR and so no antigen processing was required for presentation. Therefore any alterations in antigen processing and presentation, which may have affected the ability of cDCs to prime T cells during infection, would be missed. Future studies using whole OVA for co-cultures or making an assessment of any defect in antigen processing in cDCs using fluorescently labelled DQ-OVA could address this issue.

The limited polarisation of a Th1 response by cDCs at day 28 of infection was consistent with that seen previously [108], with both studies showing levels of TCR-transgenic T cell IFNγ production induced by cDC subsets isolated from chronically infected mice only marginally higher than that induced by cDC subsets from naïve animals. In both studies, cDC subsets have a significantly impaired capacity for IL-

12p40 (this study) and IL-12p70 [108] protein production by day 28 of infection; a defect which is likely to underlie the modest capacity for cDCs to generate functional Th1 responses by this stage of infection.

The assessment of the defect in cDC Th1 polarisation in this study was extended by including cDC / OTII cultures with the addition of a high dose of LPS. Naïve cDCs were capable of inducing a highly polarised Th1 response by OTII in the presence of LPS, whereas cDCs from chronically infected mice were unable to do so. This is consistent with the reduced ability of cDCs from day 28 infected mice to increase their production of IL-12p40 in response to LPS, resulting in far lower concentrations of IL-12 in supernatants from cDC cultures isolated from chronically infected mice and presumably far lower amounts of IL-12 available for Th1 polarisation in the co-cultures containing OTII.

In light of the dominant role for IL-27 in the generation of IL-10 producing $CD4^+$ T cells [254-257, 260, 262, 486] and the ability for cDC subsets to produce IL-27p28 protein during chronic infection determined in this study, it was surprising that cDC subsets from infected mice did not generate OTII cells capable of IL-10 production *in vitro*. A major factor in this observation could be due to dramatic differences in the amount of recombinant IL-27 used during T cell polarisation in previous studies. In this study, analysis by ELISA revealed that between 200-300 pg/ml of IL-27p28 was present in culture supernatant of cDCs isolated from chronically infected mice. This is in contrast to previous *in vitro* studies which utilised recombinant IL-27 at concentrations of 20ng/ml [255, 262, 486], 25ng/ml [259, 260], 50ng/ml [256] and 100ng/ml [254, 257], often in combination with T cell stimulation by plate-bound αCD3 and αCD28.

Such concentrations are 100 - 500 fold higher than that determined *ex vivo*. Thus it is possible that the *in vitro* polarisation assay in this study, containing physiological levels of DC-produced IL-27, were simply lacking in sufficient IL-27 to replicate the dominant polarising effect seen previously. However, High doses of IL-27 applied to cultured OTII T cells also failed to replicate the published data indicating a role for IL-27 in instructing IL-10 production, so it is possible that OTII T cells do not express sufficient WSX-1 and are thus insensitive to IL-27 signalling. Further work will be required to address this.

Although IL-10 has been shown to be a requirement for IL-10-producing regulatory 'Tr1' cell development (for example see [487]), IFNγ and IL-10 co-producing $CD4^+$ T cells of a similar phenotype to that identified here have been shown to develop via a mechanism independent of IL-10 [162]. As such the IL-10 production by cDCs may be a bigger contributor to systemic IL-10 levels during chronic infection, rather than having a role in shaping the T cell phenotype that is observed *ex vivo*. The potential role for IL-10 in directly modulating cDC function is discussed further in chapter 4 of this study.

Recent *in vitro* data has revealed key roles for IL-12 and repeated, high dose antigen stimulation in the generation of effector $CD4^+$ T cells co-producing IFNγ and IL-10 [488]. This is supported by *in vivo* studies showing that repeated exposure to antigen during chronic infection [162] and therapeutic intervention for autoimmunity [489] are required for the generation of $CD4^+Foxp3^-$ T cells capable of co-producing IFNγ and IL-10. As all cDC subsets showed severely impaired production of IL-12p40 as a result of infection, it is possible that without the production of this cytokine, OTII T cells

cannot be driven toward an IFNγ and IL-10-producing phenotype by cDCs isolated from chronically infected mice. Furthermore, antigen exposure is likely to be far higher *in vivo* than *in vitro*, suggesting that the co-cultures established in this study may have also been lacking in the required number of antigen encounters to generate IFNγ and IL-10 dual producers as seen *ex vivo*.

As there was no evidence from the co-cultures that cDCs producing IL-10 and IL-27 could generate populations of IL-10$^+$ T cells, the impact of these cytokines on T cell polarisation was determined by neutralising the function of IL-10 and IL-27 during T cell activation by cDCs *in vitro*. Blockade of IL-27 alone had very little effect on co-cultures containing cDC subsets isolated from either naive or chronically infected mice. In contrast, neutralisation of IL-10 signalling by blockade of the IL-10 receptor (IL-10R) had a marked effect. Although this was evident in co-cultures containing cDCs from naïve mice, the effect were far more limited, with neutralisation of IL-10 signalling only modestly enhancing the Th1 polarising capacity of this subset. In contrast, blockade of IL-10 signalling allowed cDC subsets from chronically infected mice to much more efficiently polarise OTII T cells toward an IFNγ$^+$ phenotype. This was accompanied by an augmented improvement in Th1 polarising capacity when IL-27 was neutralised alongside IL-10R blockade, the potential significance of which will be addressed in the next chapter.

In conclusion, the data presented in this chapter yield greater insight into the functional alterations within the CD4$^+$ T cell and cDC compartments as a result of chronic *Leishmania donovani* infection. The potential mechanisms by which the novel pattern of cDC cytokine production mediate the effects of chronic infection, as well as the role

of cDC subsets in the establishment and maintenance of chronic disease *in vivo*, will be addressed in the subsequent chapter

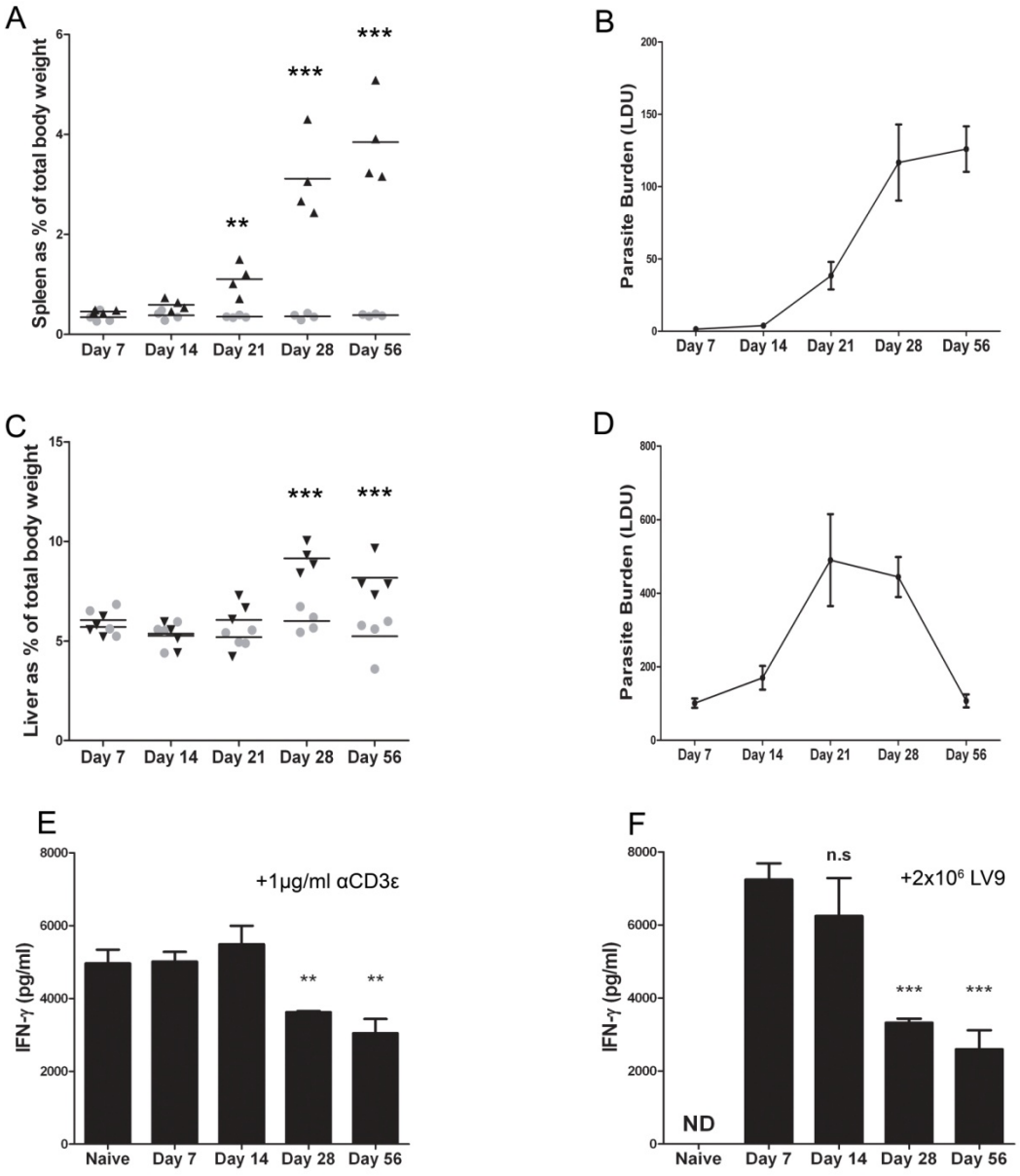

Figure 3.1 Progressive experimental visceral leishmaniasis is associated with impaired splenic IFNγ production. Female C57BL/6 mice were infected i.v with $3x10^7$ *Leishmania donovani* LV9 amastigotes. At the indicated time point post infection, mice were killed by cervical dislocation and organs isolated. **A, C**. Hepatosplenomegaly was assessed by expressing spleen and liver mass as a percentage of total body weight. **B, D**. Splenic and hepatic parasite burdens were determined from Giemsa stained impression smears. Parasite burden is expressed as Leishman-Donovan Units (LDU). Splenocytes were isolated at each time point and cultured for 72 hours with1µg/ml αCD3ε (**E**) or $2x10^6$ fixed LV9 amastigotes (**F**). Supernantants were assessed by ELISA for presence of IFNγ. Data are expressed as mean ± SEM of IFNγ production (pg/ml) from total n=8 (naïve mice) or n=4 (infected mice per timepoint). Splenomegaly and parasite burden from n=4 mice per timepoint (naïve and infected) ND = not detectable. n.s. = not significant, **= $p<0.01$, ***=$p<0.001$ for indicated day p.i compared to naïve (αCD3ε-stimulated) or day 7 infected (LV9-stimulated) mice. Or infected vs naïve groups (hepatosplenomegaly). Triangles = infected mice, circles = naïve mice.

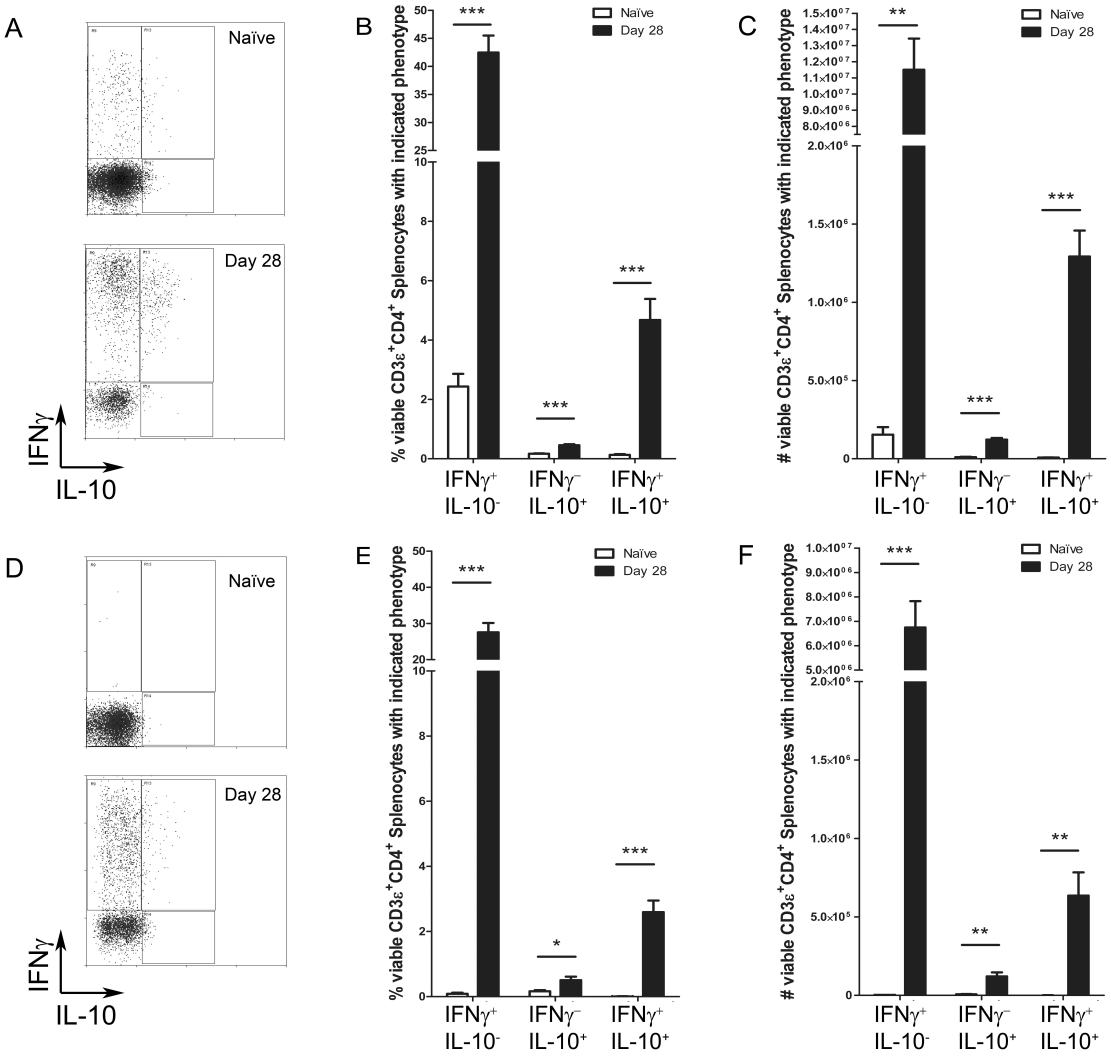

Figure 3.2 IL-10 producing CD4$^+$ T cells expand in frequency and number during chronic *Leishmania donovani* infection. Female C57BL/6 mice were infected i.v with 3x10^7 *Leishmania donovani* LV9 amastigotes and disease allowed to develop for 28 days. **A –F**. Splenocytes from infected or naïve age-matched control mice were restimulated for 2 hours with PMA and Ionomycin (**A-C**) or for 3 hours with LV9-pulsed BMDC (**D-F**) and subsequently assessed by intracellular cytokine staining for alterations in CD4$^+$ T cell phenotype. **A** and **D** show representative FACS plots, gated on CD3ε$^+$CD4$^+$ cells. **B** and **E** show the mean frequency of CD3ε$^+$CD4$^+$ T cells ± SEM with the indicated cytokine phenotype. **C** and **F** show the mean number of CD3ε$^+$CD4$^+$ T cells ± SEM with the indicated cytokine phenotype. Splenocytes from naïve (open bars) or day 28 infected mice (closed bars) are shown. Data are from n=4 mice and representative of 3 individual experiments. *=p<0.05, **=p<0.01, ***=p<0.001 for infected vs naïve mice.

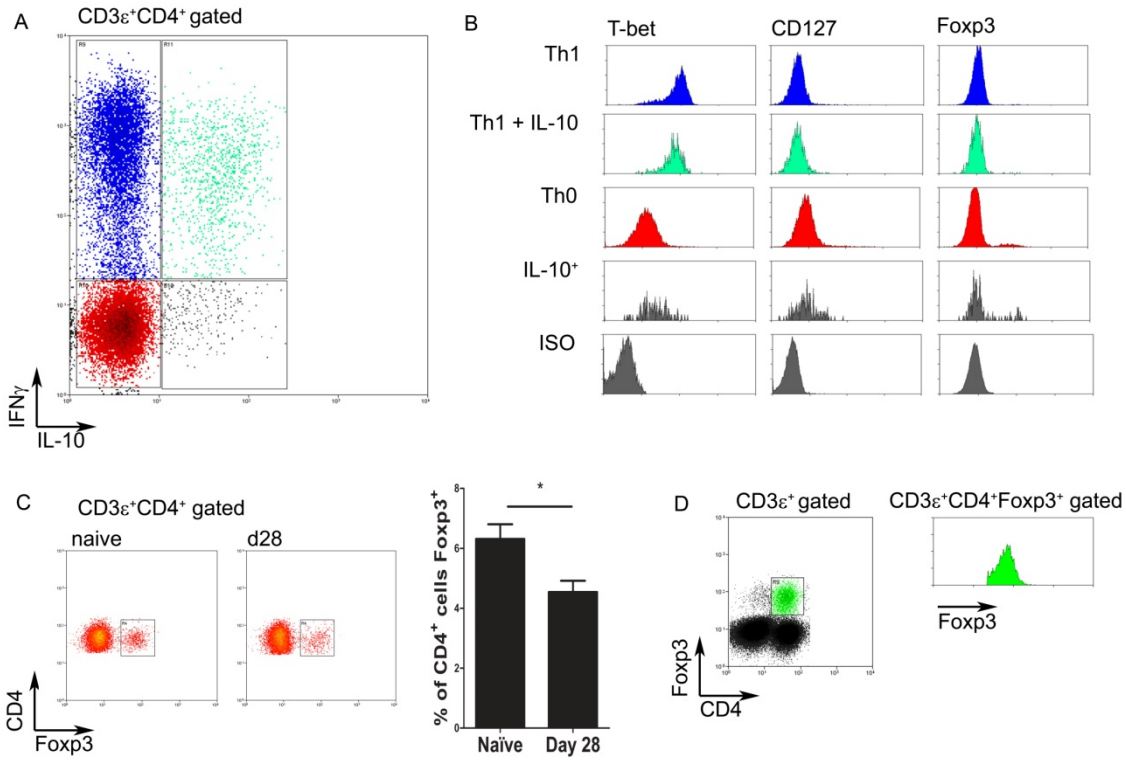

Figure 3.3 T-bet$^+$ Th1 cells are the predominant IL-10-producing T cell population during chronic infection. **A.** CD3ε$^+$CD4$^+$ T cells are capable of producing IL-10 and IFNγ during chronic infection. **B.** Populations of Th1 (IFNγ$^+$, blue), Th1 + IL-10 (IFNγ$^+$IL-10$^+$, turquoise), Th0 (no cytokine production, red) and IL-10$^+$ (grey) were assessed by flow cytometry for intracellular expression of T-bet and Foxp3 and surface expression of CD127. **C.** The frequency of splenic CD4$^+$Foxp3$^+$ natural Tregs were determined by flow cytometry in naïve and day 28-infected mice. **D**. MFI of Foxp3 expression in natural Tregs. Data shown are representative flow plots and histograms and mean frequency (±SEM) from n=3 mice. Representative of 2 experiments. *=$p<0.05$ for infected vs naïve mice.

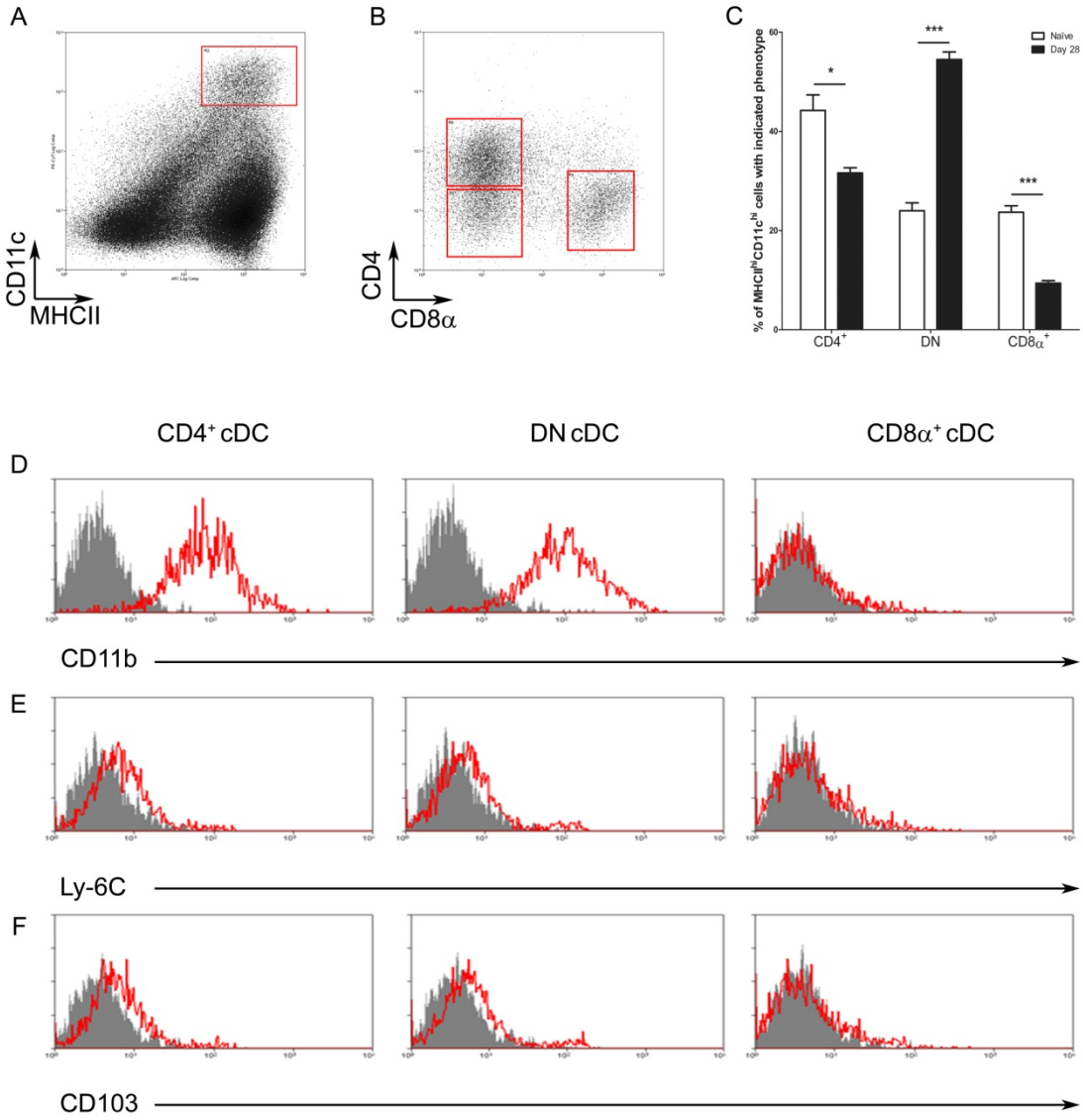

Figure 3.4 Altered composition and phenotypic analysis of the CD11c^{hi} cDC compartment during chronic infection. **A, B.** CD11c^{hi}MHCII^{hi} conventional DC can be phenotypically divided into three subsets based upon surface expression of CD4, CD8α or neither. **C.** The frequency of each cDC subset was quantified in naïve and day 28-infected mice. **D-F** cDC subset expression of CD11b, Ly6C and CD103 was determined in mice at day 28 of infection. Red line indicates specific staining, grey histogram isotype control. Data show representative dot plots or histograms, and the mean frequency of each splenic cDC subset ±SEM from n=4 mice. Representative of 2 (**D – F**) or 4 (**A – C**) individual experiments. *=p<0.05, ***=p<0.001 for infected vs naïve mice. Filled grey histogram- Isotype control, open red line- specific staining.

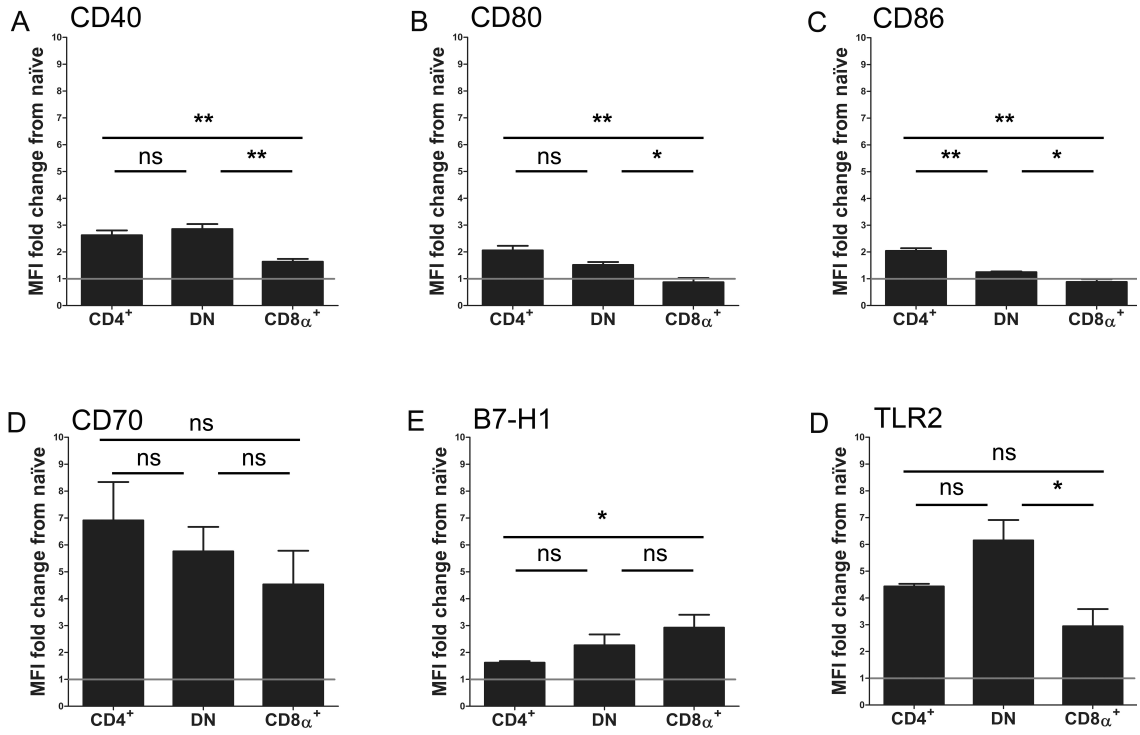

Figure 3.5 Subset-specific alterations in costimulatory molecule and TLR2 expression by splenic cDCs during chronic infection. cDC subsets from naïve or day 28 infected mice were assessed by flow cytometry for surface expression of CD40 (**A**), CD80 (**B**), CD86 (**C**), CD70 (**D**), B7-H1 (**E**) and TLR2 (**F**). Charts show mean fold increase ±SEM of surface protein expression (MFI) on the indicated $CD11c^{hi}$ $MHCII^{hi}$ cDC subset from infected mice over cDC from naïve, age matched control animals. Data are representative of 2 individual experiments, n=3-4 mice per experiment. *=$p<0.05$, **=$p<0.01$, ns = not significant for differences between cDC subsets

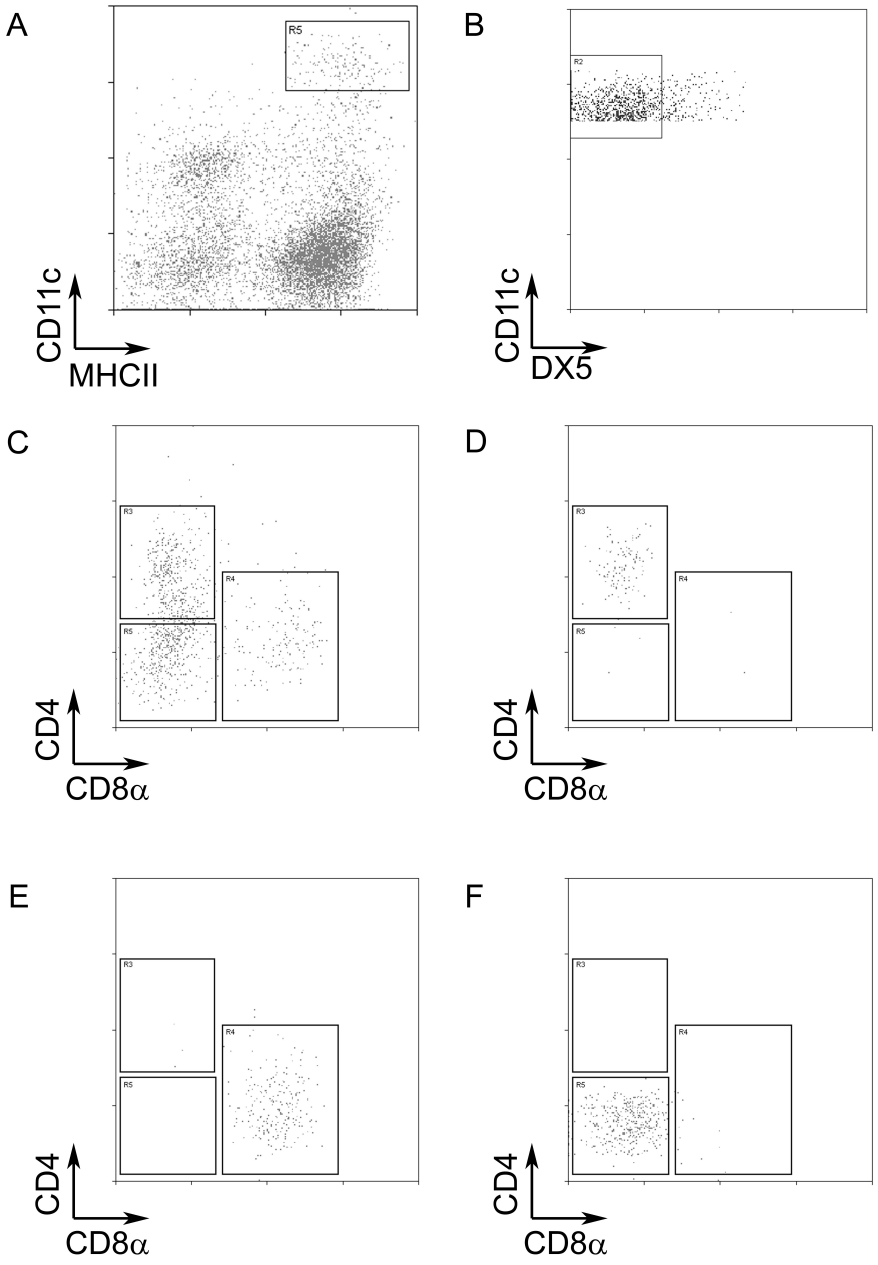

Figure 3.6 cDC subsets sorted to high purity from spleens of naïve and chronically infected mice. Conventional splenic DC were characterised by flow cytometry as $CD11c^{hi}$ $MHCII^{hi}$ (**A**) $DX5^{-}$ (**B**) and defined as three discrete subsets based upon surface expression of CD4, CD8α or neither (**C**). $CD11c^{+}$ cells were isolated by positive selection using CD11c-specific magnetic beads. cDC subsets were then flow sorted based on expression of CD4 (**D**), CD8α (**E**) or a lack of expression of both (**F**). **A** - **C** show representative FACS plots used to define parameters for sorting. **D** - **F** show representative plots of sorted populations assessed for purity by subsequent flow cytometric analysis. All cDC subset populations were routinely 98-99% pure when used for downstream analysis.

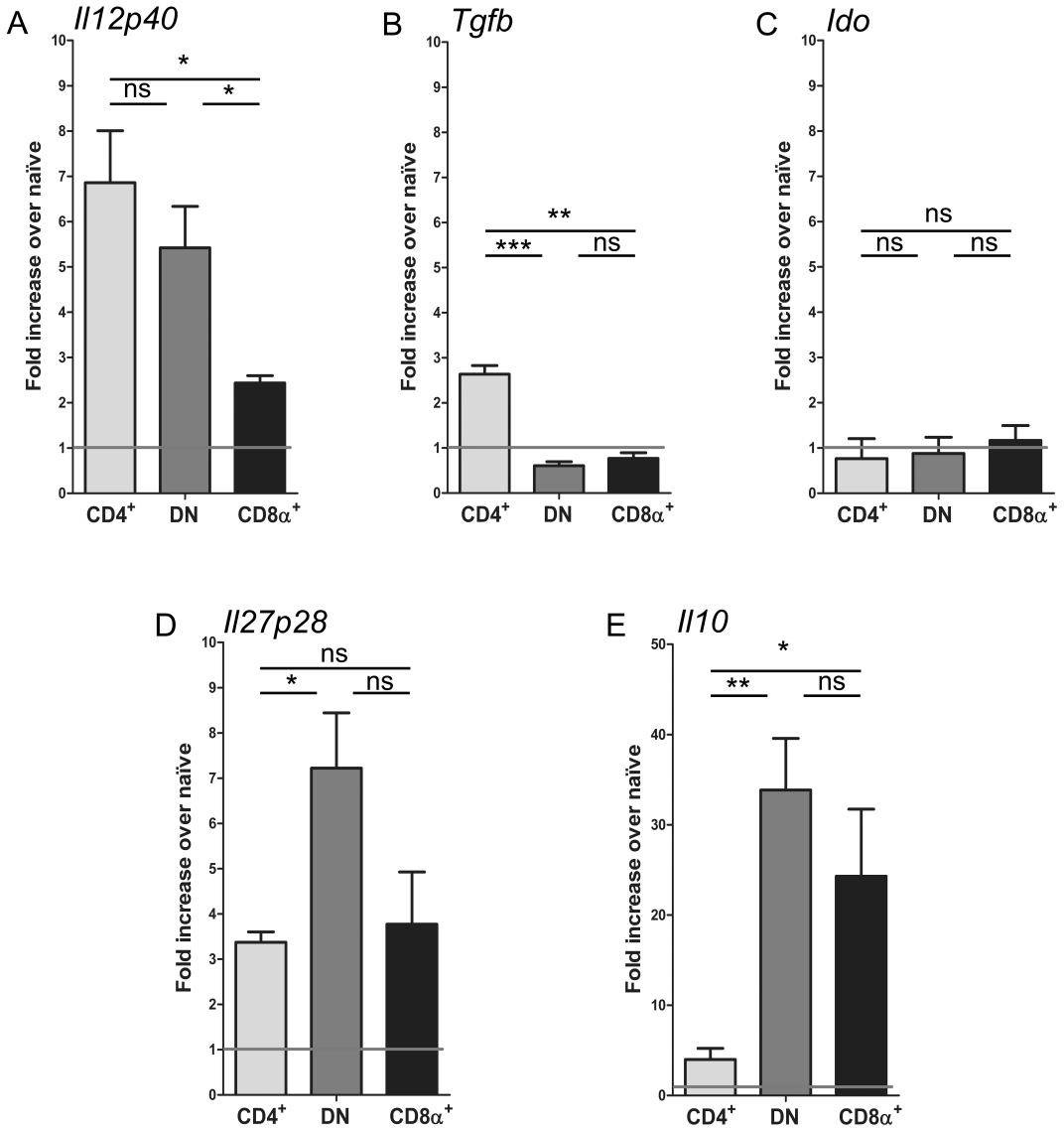

Figure 3.7 cDC subsets accumulate immunoregulatory cytokine mRNA during chronic infection. Splenic cDC subsets were sorted from individual naïve or day 28 infected mice and frozen immediately in RLT lysis and RNA stabilisation buffer. Changes in the relative expression of mRNA encoding the indicated genes between cDC from naïve or day 28-infected mice was determined by qRT-PCR, using HPRT as an endogenous control. **A - E** show fold increase in accumulation of the indicated mRNA in cDC subsets isolated from 4 individual mice at day 28 p.i, compared to cDC isolated from 4 individual naïve mice, ±SEM. Representative of two individual experiments.

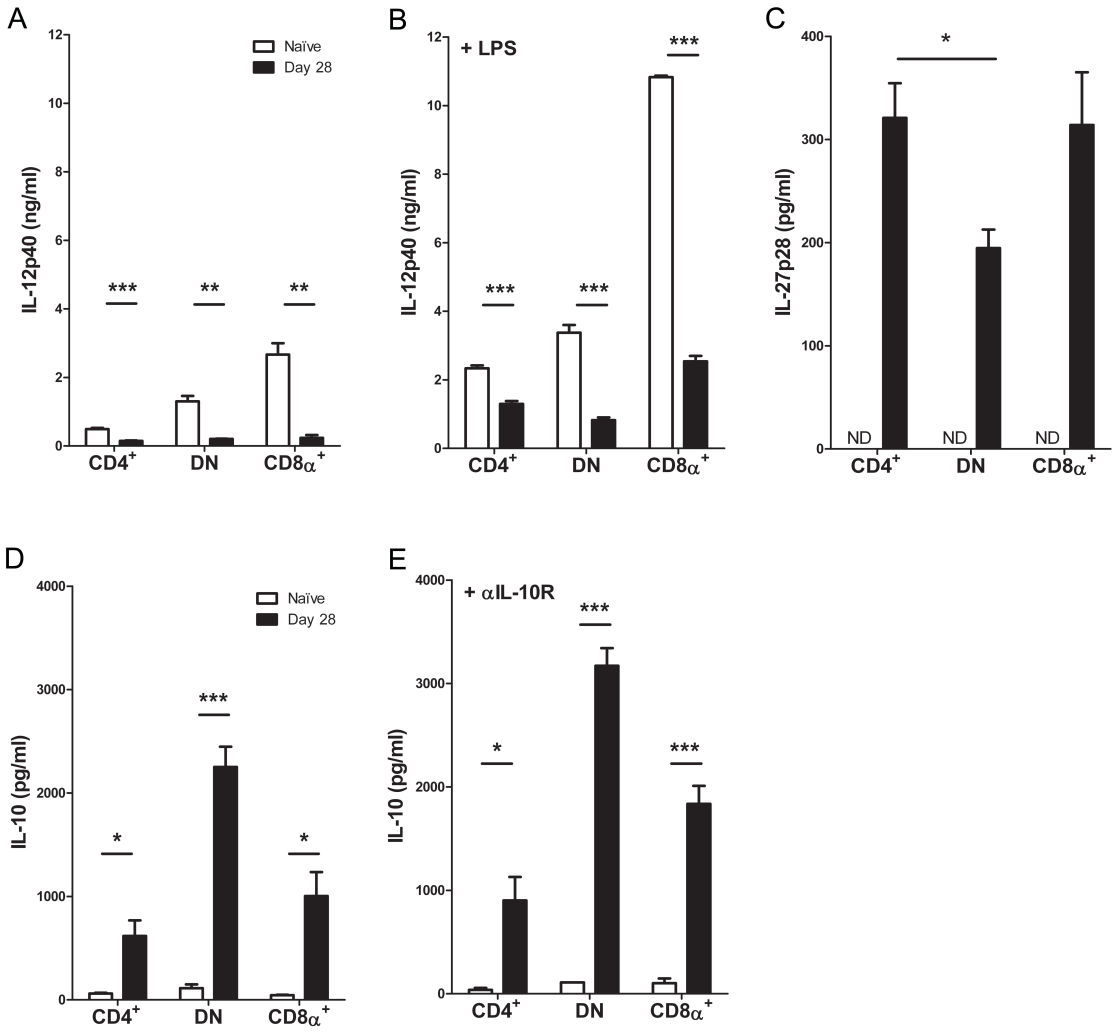

Figure 3.8 Impaired production of IL-12p40 accompanies the acquisition of an immunoregulatory cytokine profile by cDCs during chronic infection. Splenic cDC subsets were sorted from pooled naïve or individual day 28 infected mice. **A-E.** $1x10^5$ of each subset was plated, in triplicate, in complete RPMI. Cells were cultured for 24 hours at 37^oC and resultant cell supernatants assessed for the presence of IL-12p40 (**A&B**), IL-27p28 (**C**) or IL-10 (**D&E**) by sandwich ELISA, according to the manufacturer's instructions. Where indicated, cDC subsets were cultured in the presence of 1µg/ml ultrapure Lipopolysaccharide (LPS) or 1.3µg/ml anti-mouse IL-10 receptor (αIL-10R). Data shows mean production of indicated cytokine from triplicate wells of sorted cDC subsets of 6-8 pooled naïve mice (per cytokine) and sorted cDC from 3-4 individual mice at day 28 p.i. ± SEM. * = $p<0.05$, **= $p<0.01$, ***=$p<0.001$

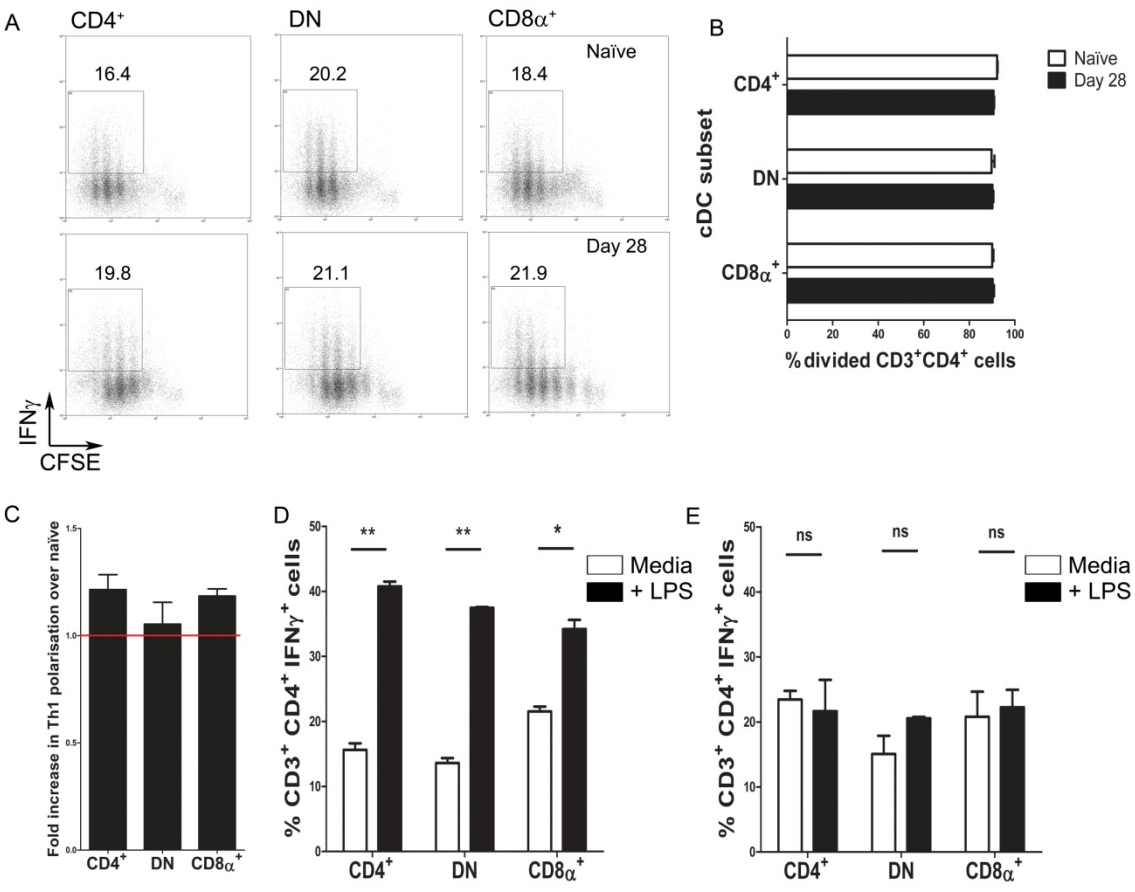

Figure 3.9 Effective CD4$^+$ T cell priming and weak Th1 polarisation by cDC subsets isolated during chronic infection. cDC subsets were sorted as previously. cDC were cultured at a 1:5 ratio with TCRβ$^+$CD44^{lo}CD62L^{hi} CFSE-labelled OTII.*Rag*2$^{-/-}$ T cells, in the presence of 5nM OVA$_{(323-339)}$ peptide. After 5 days, cells were restimulated with PMA and Ionomycin for 2 hours. **A-D**. CD3ε$^+$CD4$^+$ T cells were assessed by flow cytometry for proliferation and IFNγ production. **A** shows representative FACS plots indicating CFSE dilution and IFNγ production by CD3$^+$CD4$^+$ cells induced by cDC subsets from naïve (upper panel) or day 28 infected (lower panel) mice. **B** shows the mean level of OTII proliferation induced by the indicated cDC subset from naïve or day 28 infected mice ±SEM. **C** shows the mean fold increase in Th1 polarising capacity of cDC from chronically infected mice, when compared to cDC from naïve animals ±SEM. **D** and **E** show the capacity for LPS-stimulated cDC subsets from naïve (**D**) or day 28 infected (**E**) mice to instruct Th1 commitment in OTII T cells. Data are from 3-5 replicate wells per cDC subset and are representative of 2 individual experiments. *=p<0.05 **= p<0.01, for LPS-stimulated cultures compared to media only control wells.

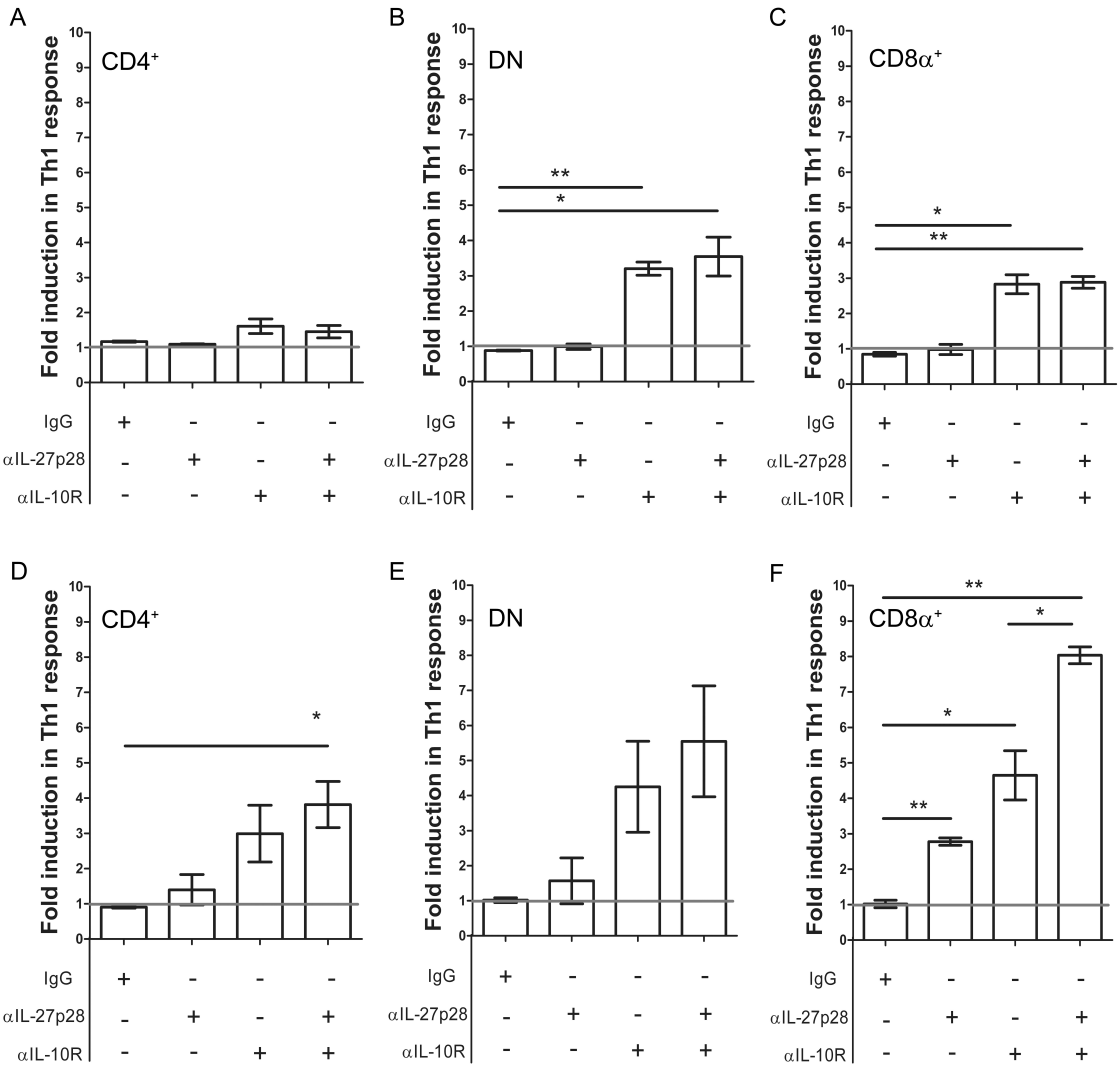

Figure 3.10 Coordinate inhibition of IL-10 and IL-27 signalling improves the Th1 polarising capacity of cDC subsets isolated from chronically infected mice. **A-E.** cDC subsets from naïve (**A–C**) or day 28 infected (**D-E**) mice and naïve OTII T cells were sorted and cultured as described, in the presence of control IgG, αIL-27p28, αIL-10R or both. Data show mean fold change in capacity for Th1 induction, determined by dividing the frequency of IFNγ^+ OTII T cells in triplicate cDC-T cell co-cultures with the indicated treatment by the frequency in triplicate cultures containing RPMI and $OVA_{(323-339)}$ only. Data are mean ±SEM, and are from one experiment. * = $p<0.05$, **= $p<0.01$

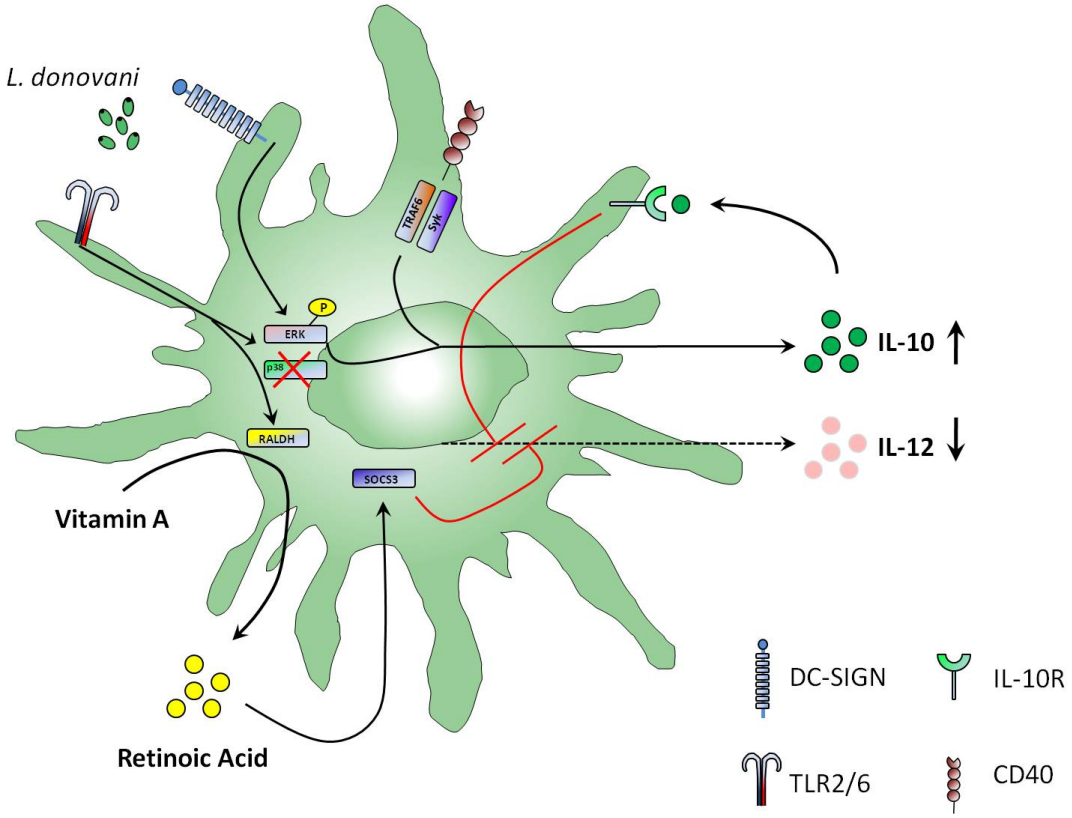

Figure 3.11 Potential mechanisms of altered cDC cytokine production due to chronic infection

Several signalling pathways may be implicated in the simultaneous enhanced IL-10 production and repressed IL-12 production by splenic cDCs observed in this study. These include i) direct recognition of *L. donovani* by TLR2 and/or DC-SIGN, leading to ERK activation and IL-10 production; ii) formation of a TRAF6 and Syk-containing signalosome downstream of CD40; iii) metabolism of retinoic acid and autocrine induction of SOCS3 and iv) autocrine uptake up of IL-10 and suppression of IL-12p40 gene induction. Adapted from [490], original literature cited in text.

Chapter 4: CD11c^{hi} cDCs facilitate the expansion of IFNγ^{+} IL-10^{+} CD4^{+} T cells *in vivo* and help maintain chronic infection with *L. donovani*

4.1 Introduction

The altered cytokine profile of splenic cDCs identified in the previous chapter suggested a potential auto-regulatory role for IL-10 and IL-27 in modulating production of IL-12 by these cells during infection. Such autocrine IL-10 signalling is known to suppress IL-12 production by DCs as a result of *Mycobacterium tuberculosis* infection [491], as well as inhibiting IL-12p70 production by splenic cDCs after TLR stimulation *in vitro* [492]. Suppression of TLR-induced IL-12 expression via activation of ERK occurs due to autocrine uptake of IL-10 by BMDCs [493] and the functional exhaustion of DCs is purportedly mediated by a similar mechanism [494]. There is no direct evidence for the inhibition of IL-12 production by DCs as a result of IL-27 signalling; however, enhanced activation of DCs from IL-27R-deficient mice [495] and the inhibition of macrophage IL-12 production by IL-27 *in vitro* [496] suggest a potential regulatory role for this cytokine at the level of the APC. To date, no study has addressed the role for autocrine IL-10 or IL-27 signalling in modulating the cytokine profile of splenic cDCs isolated during chronic parasitic infection *in vivo*.

IL-27 production by DCs and macrophages is known to be dependent on several elements of the type I interferon (IFN) pathway. IFNα is critically required for expression of the IL-27p28 subunit in TLR3, 4 or 7/8-stimulated human macrophages, as a direct result of IRF1 induction and subsequent binding to an ISRE in the p28 gene promoter [497]. Confirming this crucial role for IRF1, stimulation of human monocyte-

derived DCs with another type I IFN, IFNβ, either alone or in conjunction with LPS, leads to enhanced IL-27p28 expression, again as a result of IRF1 activation [498]. IRF3 has been proposed as a 'master switch' for IL-27 expression, with *Irf3*$^{-/-}$ BMDCs showing reduced IL-27p28 production in response to LPS, *Irf3*$^{-/-}$ mice having lower concentrations of serum IL-27 in response to LPS administration and the existence of an IRF3-specific binding site within the human IL-27p28 promoter [499].

More recently, a sequential process leading to IL-27p28 expression has been proposed, whereby in response to TLR3 or TLR4 stimulation, initial IL-27p28 expression is induced by IRF1 and IRF3 and the subsequent amplification of expression is dependent upon recruitment of an IRF9-containing IFN-stimulated gene factor 3 complex [500]. This is further complicated by a role for IRF8 in enhancing IL-27 production by macrophages as a result of direct interaction with the p28 promoter at a binding site which overlaps with that of IRF1 [501]. Although BMDCs from B6.*Irf7*$^{-/-}$ mice show normal IL-27 production in response to LPS [500], it is not known whether IRF7 is required for production of IL-27 by splenic cDCs in response to infection *in vivo*. In addition, as studies addressing a function for IRF7 in the response to *Leishmania donovani* infection *in vivo* have been restricted to the acute stages of parasitisation [502] and chronic infection of the liver (Beattie *et al*, in press), little is known about the role for this transcription factor in the establishment of chronic disease in the spleen.

The ability to selectively deplete CD11c-expressing dendritic cells was first established using transgenic animals carrying a simian diphtheria toxin receptor (DTR) under control of the CD11c promoter [503]. This system allowed for conditional ablation of dendritic cells in an otherwise intact animal after administration of diphtheria toxin

(DTx). However, toxicity as a result of repeated DTx treatment limited studies with this original line of mice to short-term DC ablation, unless bone marrow radiation chimeras were generated [504].

In addition, new strains of mice have been developed that allow for multiple injections of DTx, and therefore sustained DC ablation, including CD11c.DOG mice bearing a human DTR under the control of the CD11c promoter [505]. Studies using this line of mice established the feasibility of sustained depletion of splenic $CD11c^+$ cells for up to 11 days during *Schistosoma mansoni* infection [506]; a chronic disease associated with splenic enlargement of a similar order of magnitude to that seen in *L. donovani*-infected C57BL/6 mice.

In order to avoid issues with DTx toxicity, a novel strategy for CD11c-depletion during chronic infection was employed in this study, using mice in which *Cre* recombinase is expressed under control of the CD11c promoter [507], crossed with mice bearing a *loxP*-flanked STOP cassette upstream of a simian DTR sequence [508]. As such, the resulting strain has DTR expression restricted to cells in which CD11c promoter activity has occurred. Very few studies have attempted to address the role of DCs in *Leishmania* infection by such conditional ablation approaches. These have been limited to short-term DC depletion after acute infection with *L. infantum* promastigotes [353]; transient ablation of DCs during *L. donovani* and *Streptococcus pneumoniae* co-infection [509]; and depletion of langerin-expressing cutaneous DC populations during *L. major* infection using a langerin-DTR ablation system [510].

This chapter therefore aims to elucidate the mechanisms behind the dysregulated cytokine profile of cDCs, to assess any role for IRF7 in IL-27 production and pathology during infection and for the first time determine the impact of *in vivo* dendritic cell ablation on disease progression and pathology during chronic infection with *L. donovani*.

4.2 Results

4.2.1 Autocrine IL-10 signalling inhibits IL-12p70 production by cDCs

In order to gain an insight into the potential mechanisms responsible for the altered cDC cytokine profile of identified during chronic infection, total $CD11c^{hi}MHCII^{hi}$ splenic cDCs were sorted from groups of naïve and day 28 infected C57BL/6 mice and cultured for 24 hours in the presence of LPS, control IgG, αIL-27p28 and αIL-10R, or combinations thereof, before levels of IL-12p70 (**4.1A&B**) and IL-10 (**4.1C&D**) in culture supernatants were determined by ELISA.

cDCs isolated from naïve and infected mice showed very low levels of IL-12p70 production when cultured in the absence of exogenous stimulation. Upon LPS stimulation, cDCs from naïve mice produced significantly more IL-12p70, increasing from 2.54 ±0.47pg/ml from unstimulated cDCs to 28.36 ±5.59 pg/ml ($p<0.05$) in the presence of LPS. Although barely detectable *ex vivo*, IL-12p70 production by cDCs from day 28 infected mice increased to 12.26 ±1.65 pg/ml ($p<0.01$) when cultured with LPS. Adding Isotype control (IgG) or αIL-27p28 antibodies in the presence or absence of LPS during culture had no effect on IL-12p70 production by cDCs from naïve or infected mice, with levels of IL-12p70 in supernatant not significantly altered at 3.54 ±0.22 and 3.77 ±0.13 pg/ml, respectively. In contrast, inhibition of IL-10 signalling by culture with an IL-10R blocking antibody had a profound effect on cDC production of IL-12p70, particularly by cDCs isolated from chronically infected mice.

IL-10R blockade in isolation had a small but significant effect on IL-12p70 production by cDCs isolated from naïve mice, with levels in supernatant increasing to 7.16 ±1.04

pg/ml ($p<0.05$) after culture. Similarly, addition of αIL-10R to cDCs isolated from mice at day 28 of infection led to an increase in IL-12p70 production to 9.05 ±0.83 pg/ml ($p<0.01$) after 24 hours of culture. When inhibition of IL-10 signalling was combined with LPS stimulation, IL-12p70 production by cDCs from both naïve and infected mice was dramatically increased. This was represented by increases to 101.41 ±20.37 pg/ml ($p<0.01$) and 266.11 ±7.79 pg/ml ($p<0.0001$) of IL-12p70 in culture supernatant of cDCs from naïve and day 28 infected mice, respectively when compared to cDCs cultured in the presence of LPS and IgG. Blockade of IL-10R alongside neutralisation of IL-27p28 during culture led to significantly higher production of IL-12p70 by cDCs isolated from both naïve and infected mice, when compared to those cultured with IgG, at 6.14 ±0.74 ($p<0.05$) and 7.03 ±0.89 pg/ml ($p<0.01$), respectively. However, the combined inhibition of IL-10 and IL-27 signalling did not have a supplementary effect on IL-12p70 production by cDCs with levels similar to those observed when IL-10 signalling was blocked in isolation. A similarly enhanced response was observed when cDCs were cultured with IL-10R blocking and IL-27p28 neutralising antibodies in the presence of LPS. This was reflected by highly significant increases in IL-12p70 production, reaching 87.83 ±6.25 and 269.53 ±8.21pg/ml in culture supernatants of cDCs isolated from naïve and chronically infected mice respectively. Again, enhanced production of IL-12p70 by cDC when IL-10 and IL-27 signalling were both inhibited was not significantly greater than that observed when IL-10R was blocked in isolation.

A similar approach was taken to assess whether IL-10 production by cDCs isolated from naïve or chronically infected mice was affected by IL-10R blockade and IL-27p28 neutralisation. After 24 hours in culture, culture supernatant from cDCs isolated from naïve animals had very low levels of IL-10 (**4.1C**). IL-10 was only detectable in culture

supernatant when cDCs were cultured in the presence of αIL-10R and LPS or αIL-10R, αIL-27p28 and LPS (62.23 ±9.2 and 55.07 ±4.55 pg/ml, respectively).

In contrast to those isolated from naïve mice, cDCs sorted from mice at day 28 of infection were capable of IL-10 production even in the absence of exogenous stimulation (**4.1D**). The addition of LPS augmented this IL-10-producing capacity, with levels increasing from 324.29 ± 19.54 pg/ml in supernatant of cDCs cultured in media alone to 583.48 ±32.84 pg/ml ($p<0.01$) when cultured in the presence of LPS. Addition of control IgG or αIL-27p8, alone or in combination with LPS, had no significant effect on levels of IL-10 production by cDCs, when compared with respective control treatments. However, culture with an IL-10R blocking antibody enhanced IL-10 production by cDCs, reflected by an increase in IL-10 from 345.15 ±55.50 pg/ml in supernatant of cDCs cultured with IgG to 889.11 ±60.36 pg/ml ($p<0.01$) when cultured with αIL-10R. Similarly, addition of αIL-10R to cDC cultures in the presence of LPS dramatically augmented the IL-10-producing capacity of cDCs, with levels increasing to 1953.93 ±118.60 pg/ml in culture supernatant after 24 hours. Combined inhibition of IL-10 and Il-27 signalling, in the presence or absence of LPS, had a very similar effect on cDC IL-10 production as IL-10R blockade alone. In the absence of LPS, cDC IL-10 production increased to 760.52 ±97.58 pg/ml ($p<0.05$) and when LPS was added IL-10 levels in culture supernatant reached 1904.02 ±31.92 pg/ml ($p<0.01$). Collectively, this confirmed that IL-10 is produced and taken up by cDCs as a result of chronic infection and suggests that this IL-10 may contribute to the inhibition of IL-12p70 production by cDCs at this stage of infection.

4.2.2 IL-27 inhibits IL-12 mediated Th1 induction *in vitro* by an IL-10-dependent mechanism

As a negative effect of cDC-derived IL-27 on *in vitro* T cell polarisation had been suggested by earlier data (cf. Chapter 3) and neutralisation of IL-27p28 had little effect on cDC cytokine production (**4.1**), to address the potential role of IL-27 production by cDCs, the impact of exogenous IL-27 on $CD4^+$ T cell polarisation was determined *in vitro*.

Total splenic $CD11c^{hi}$ cDCs were sorted from naïve C57BL/6 mice and cultured with sorted naïve OTII.*Rag*$2^{-/-}$ T cells, in the presence of $OVA_{(323-339)}$ and combinations of the indicated cytokines (**4.2A&B**). After 5 days of culture, cells were restimulated and $CD3\varepsilon^+CD4^+$ OTII T cells assessed for functional polarisation by intracellular cytokine staining and flow cytometry. Restimulation with PMA and Ionomycin revealed that OTII T cells were solely capable of IFNγ production (**4.2A**). In the absence of exogenous cytokines, cDCs induced 7.34 ±0.89% of OTII to become $IFN\gamma^+$ (4.2*B*), presumably due to spontaneous activation of splenic cDCs after isolation and culture (Owens, unpublished observations and [511]). This level of polarisation was significantly reduced to 4.5 ±0.22% ($p<0.05$) when OTII were polarised in the presence of IL-27. As expected, addition of IL-12 to the cultures generated a potent Th1 response, with $IFN\gamma^+$ OTII cells increasing in frequency to 17.91 ±0.29% after restimulation. Co-culture of cDCs and OTII in the presence of both IL-12 and IL-27 significantly impaired IL-12-induced Th1 polarisation, with the frequency of $IFN\gamma^+$ OTII T cells generated reduced to 7.96 ±0.45% ($p<0.0001$).

In order to determine a potential mechanism for the observed inhibition of Th1 polarisation by IL-27, total splenic cDCs were sorted from naïve C57BL/6 and B6.*Il10*$^{-/-}$ mice and co-cultured with sorted naïve OTII T cells in the presence of cytokines as before (**4.2C**). Although frequencies of viable cells as a whole were slightly lower in this experiment, activation of OTII with C57BL/6 cDCs in the presence of IL-27 again led to a significant decrease in the percentage of IFNγ^{+} OTII cells from 2.36 ±0.12% when activated in media alone to 1.68 ±0.08% ($p<0.01$) when supplemented with IL-27. The addition of IL-12 again led to enhanced Th1 polarisation, with 6.50 ±0.19% ($p<0.0001$) OTII expressing IFNγ after culture. Addition of IL-27 suppressed this Th1 induction, with IFNγ^{+} OTII significantly reduced in frequency to 4.08 ±0.35% ($p<0.01$) when these cytokines were combined.

In contrast, co-cultures containing splenic cDCs from IL-10-deficient mice did not contain a lower frequency of IFNγ^{+} OTII when cultured with IL-27, with equivalent frequencies of IFNγ^{+} OTII at 2.89 ±0.37% with media alone and 2.52 ±0.27% in the presence of IL-27. The IL-12-induced polarisation of OTII cells by *Il10*$^{-/-}$ cDCs was slightly enhanced compared to wildtype cDCs, with 7.95 ±0.73% of OTII capable of IFNγ^{+} production after restimulation. However, the ability for IL-27 to inhibit IL-12-induced IFNγ production by OTII was abolished when *Il10*$^{-/-}$ cDCs were used as APCs, as reflected in a frequency of IFNγ^{+} OTII generated in the presence of IL-12 and IL-27 of 7.22 ±0.34%, not significantly altered from the frequency when stimulated with IL-12 alone. This therefore suggests that the capacity for IL-27 to impair IL-12-induced Th1 polarisation is, at least in part, dependent on cDC-derived IL-10.

4.2.3 IRF7 is not required for cDC IL-27 production *in vitro* or *in vivo* and splenic responses to chronic *L. donovani* infection are unaffected in B6.*Irf7*$^{-/-}$ hosts

Female C57BL/6 and B6.*Irf7*$^{-/-}$ mice were infected with *L. donovani* as described above. Chronic infection was allowed to establish and mice were killed at day 28. C57BL/6 and B6.*Irf7*$^{-/-}$ animals showed equivalent alterations in spleen mass as a result of infection, with spleens comprising 3.94 ±0.52% and 4.04 ±0.27% of body weight in day 28-infected wildtype and IRF7- deficient mice, respectively (**4.3A**). Parasite burdens in spleens of chronically infected mice were also equivalent, at 105.7 ±18.7 and 121.3 ±14.7 (p=ns) LDUs in C57BL/6 and B6.*Irf7*$^{-/-}$ animals (**4.3B**)

Levels of CD40 (**4.3C**) and CD86 (**4.3D**) expression on cDC subsets were equivalent in steady state C57BL/6 and B6.*Irf7*$^{-/-}$ mice, with the MFI of CD40 expression on CD4^{+}, DN and CD8α^{+} cDC subsets at 48.19 ±0.93, 37.41 ±0.55 and 63.03 ±0.82 units in C57BL/6 mice and at 54.34 ±2.69, 38.79 ±2.04 and 70.46 ±4.62 in mice deficient in IRF7. Similarly, the MFI of surface CD86 expression on cDCs were comparable, with the CD4^{+}, DN and CD8α^{+} subsets having surface levels of 14.89 ±1.95, 17.00 ±0.44 and 22.37 ± 3.78 fluorescence units when assessed in C57BL/6 mice and 14.63 ±0.51, 10.29 ±1.93 and 18.21 ±2.37 when splenic cDCs from B6.*Irf7*$^{-/-}$ mice were assessed by flow cytometry.

Showing similar patterns to those previously observed (**3.5**), cDC subset costimulatory molecule expression on cDC subsets, particularly apparent with CD86 and on CD8α^{+} cDCs, was only modestly increased after infection. Reflecting the comparable expression levels seen in naïve mice, there were also no major differences in

costimulatory molecule expression on cDCs in chronically infected wildtype or IRF7-deficient animals. This was reflected by CD40 expression levels on $CD4^+$, DN and $CD8\alpha^+$ cDC subsets from infected wildtype mice of 126.13 ±7.42, 106.48 ±5.64 and 103.05 ±6.44 fluorescence units and 106.90 ±6.48, 118.14 ±9.50 and 107.70 ±7.92 units on the surface of cDC subsets in spleens of B6.*Irf7*$^{-/-}$ animals. Similarly, CD86 expression on cDC subsets during chronic infection was equivalent in C57BL/6 and B6.*Irf7*$^{-/-}$ mice. $CD4^+$, DN and $CD8\alpha^+$ cDCs expressed CD86 at 30.01 ±2.40, 21.22 ±0.90 and 19.00 ±0.84 units in C57BL/6 mice and 28.60 ±2.44, 20.35 ±0.54 and 19.19±1.28 in spleens of B6.*Irf7*$^{-/-}$ mice chronically infected with *L. donovani*, indicating that IRF7 deficiency does not substantially alter the regulation of costimulatory molecule expression on cDCs in the steady state or during inflammation.

As various members of the IRF family have been implicated in the regulation of IL-27 production [497-501], it was of interest to determine whether cDCs deficient in IRF7 were impaired in their capacity to produce IL-27 as a result of chronic of infection (**4.3E**). Total cDCs were sorted from spleens of naïve and day 28 infected C57BL/6 and B6.*Irf7*$^{-/-}$ mice and cultured for 24 hours in the absence of exogenous stimulation. In agreement with previous data obtained from sorted cDC subsets (cf. Chapter 3), chronic infection of C57BL/6 mice resulted in significantly enhanced levels of IL-27 production by cDCs, with mean IL-27 levels in culture supernatant of 43.54 ±33.33 pg/ml after culture of cDCs from naïve mice and 403.80 ±23.90 pg/ml ($p<0.0001$) after culture of cDCs sorted from day 28 infected mice. Chronic infection of IRF7-deficient mice also led to a significantly enhanced level of IL-27 production by cDCs, with IL-27p28 in culture supernatant at 26.56 ±12.40 pg/ml and 283.84 ±37.08 pg/ml in culture supernatant of cDCs isolated from naïve and day 28 infected mice, respectively.

Although slightly lower than cDCs isolated from C57BL/6 mice, a lack of IRF7 did not impact significantly upon the ability of cDCs to produce IL-27 as a result of chronic infection.

To establish whether IRF7 deficiency impacted on systemic immune responses in chronically infected mice, serum was isolated from peripheral blood of naïve and day 28 infected C57BL/6 and B6.*Irf7*$^{-/-}$ mice. Levels of IL-12p70 (**4.3F**) and IL-27p28 (**4.3G**) were determined by ELISA. Although elevated in infected mice of both strains, serum IL-12p70 levels were significantly increased only in B6.*Irf7*$^{-/-}$ mice as a result of chronic infection (3.95 ±0.48pg/ml vs 9.97 ±1.78pg/ml ($p<0.05$) in sera from naïve and infected B6.*Irf7*$^{-/-}$ mice, respectively). IL-27p28 was barely detectable in sera from naïve mice of either strain. Chronic infection led to a significant increase in serum IL-27p28 levels from 2.60± 1.50 to 341.85 ±96.40pg/ml ($p<0.05$) in wildtype mice and 6.78 ±1.68 to 272.82 ±16.98 pg/ml ($p<0.001$) in IRF7-deficient animals. Although slightly lower in B6.*Irf7*$^{-/-}$ mice, there was no significant difference between serum IL-27p28 levels between the two strains at day 28 of infection.

Finally, to investigate any potential role for IRF7 in IL-27p28 production by cDCs in response to other stimuli, cDCs were sorted from naïve C57BL/6 and B6.*Irf7*$^{-/-}$ mice and cultured for 24 hours in the presence of LPS or IFNα, before IL-27p28 levels in culture supernatant were determined by ELISA (**4.3H**). cDCs from both strains produced very little IL-27p28 when cultured for 24 hours in the absence of exogenous stimulation. Addition of LPS increased IL-27 production to 61.23 ±3.29pg/ml by wildtype cDCs and 59.00 ±0.75pg/ml by B6.*Irf7*$^{-/-}$ cDCs, with no significant difference in levels between the strains. Addition of IFNα strongly enhanced IL-27p28 production by cDCs from

both strains, with levels of IL-27 production significantly higher (216.92 ±5.60pg/ml) in culture supernatant from IRF7-deficient cDCs compared to those from wildtype mice (124.18 ±4.79pg/ml; $p<0.001$). These data indicate that IRF7 is not essential for IL-27 production by splenic cDCs *in vitro* or *in vivo*, and that IRF7 may regulate production of IL-27 in response to exogenous IFNα.

4.2.4 Equivalent T cell responses in chronically infected mice deficient in IRF7

To determine whether IRF7 deficiency impacted upon T cell polarisation during chronic infection, splenocytes from naïve and infected C57BL/6 and B6.*Irf7*$^{-/-}$ mice were restimulated *in vitro* in the presence of PMA, Ionomycin and Brefeldin A (**4.4**). After restimulation, cytokine production by T cells was assessed by ICS and flow cytometry, with T cells gated as viable, CD3ε^{+} and expressing either CD4 or CD8α (**4.4A**). The frequency of CD4^{+} and CD8α^{+} T cells was equivalent in both strains (See chapter 5.1 for details). CD8α^{+} (**4.4B&D**) and CD4^{+} (**4.4C&E**) T cells were assessed for expression of intracellular IFNγ and IL-10. Production of IFNγ by CD8α^{+} T cells after PMA and Ionomycin stimulation of naïve splenocytes was equivalent in both strains at 17.03 ±0.81 and 14.45 ±0.35% in C57BL/6 and B6.*Irf7*$^{-/-}$ mice, respectively (**4.4D**). IFNγ production by CD8α^{+} T cells increased as a result of chronic infection, but there was no significant difference in the frequency of these cells at 50.64 ±4.36 and 42.3 ±1.53% in spleens of chronically infected wildtype and IRF7-deficient mice.

The frequency of CD4^{+}IFNγ^{+} T cells in naïve B6.*Irf7*$^{-/-}$ mice was significantly elevated compared to wildtype animals, at 3.36 ±0.34% of splenocytes compared to 1.45 ±0.10% ($p<0.01$) in spleens of C57BL/6 mice (**4.4E**). CD4^{+}IL-10^{+} T cells were at an equivalent

frequency in steady state B6.*Irf7*$^{-/-}$ and C57BL/6 mice, at 0.70 ±0.07 and 1.33 ±0.26%, respectively. CD4^{+} T cells producing both IFNγ and IL-10 were at very low and equivalent levels of 0.20 ±0.01 and 0.40 ±0.10% in wildtype and B6.*Irf7*$^{-/-}$ mice.

Consistent with previous observations, infection with *L. donovani* led to the expansion of CD4^{+} T cell populations expressing IFNγ, IL-10 or both. A lack of IRF7 did not significantly affect the frequency of CD4^{+} T cells capable of IFNγ production, with 20.06 ±5.31 and 17.96 ±2.19% of CD3ε^{+}CD4^{+}IFNγ^{+} cells in spleens of C57BL/6 and B6.*Irf7*$^{-/-}$ mice by day 28 of infection. Splenic CD4^{+}IL-10^{+} T cells were at a significantly higher frequency in chronically infected B6.*Irf7*$^{-/-}$ mice, representing 2.70 ±0.34% of splenocytes in these animals, compared to 1.17 ±0.09% ($p<0.05$) in chronically infected C57BL/6 mice. CD4^{+}IFNγ^{+}IL-10^{+} T cells expanded dramatically in frequency as a result of infection, comprising 4.16 ±1.54% in wildtype mice and 4.78 ±0.50% (p=ns) in IRF7-deficient mice by day 28 of infection. Taken together, IRF7 deficiency did not significantly alter the splenic effector T cell response during chronic infection, with only a slight, albeit significant, enhancement in T cell IL-10 production being observed in these mice.

4.2.5 Efficient conditional ablation of CD11c^{+} cells in CD11c.iDTR mice

To assess the functional impact of the altered dendritic cell phenotype characterised in chapter 3, studies were performed with mice in which the conditional ablation of CD11c-expressing cells is facilitated by injection of Diptheria Toxin (DTx) (**4.5**). Mice in which expression of *Cre* recombinase is dependent on CD11c-promoter activity (**4.5Ai**) were crossed with mice in which a construct containing a *loxP*-flanked STOP

cassette upstream of a simian DTR sequence is inserted into the ubiquitously expressed ROSA26 locus (**4.5Aii**). Past or current expression of *Cre* within $CD11c^{+}$ cells results in cleavage at *loxP* sites flanking the STOP cassette, leading to expression of DTR on the cell surface, effectively resulting in CD11c-dependent DTR expression (**4.5Aiii**). Such mice, designated hereafter as CD11c.iDTR, enable depletion of CD11c-expressing cells and cells with past expression of CD11c by administration of DTx by i.p injection.

To confirm the utility of these mice, the effects of DTx administration to naïve mice was determined by flow cytometry. Importantly, despite injection of multiple (4) doses of DTx at 48hr intervals over a 7 day period, no lethal toxicity was observed in any group of CD11c.iDTR mice, as has been reported for certain original strains of CD11c-DTR animals [512]. Administration of DTx to naïve animals led to substantial decreases in the number of splenic CD11c-expressing cells (**4.5B-C**). This included i) a slight reduction in the number of $CD11^{int}MHCII^{-}$ cells, from $1.32x10^{6}$ $\pm 2.33x10^{4}$ in PBS treated animals to $1.01x10^{6}$ $\pm 1.23x10^{5}$ after DTx administration; ii) a significant loss of $CD11c^{int}MHCII^{hi}$ cells, decreasing in number from $9.33x10^{5}$ $\pm 4.97x10^{4}$ in PBS treated mice to $4.98x10^{5}$ $\pm 8.51x10^{4}$ ($p<0.05$) cells after administration of DTx; and iii) highly significant ablation of $CD11c^{hi}MHCII^{hi}$ cDCs, with depletion of almost 90% of these cells. cDC numbers dropped from $1.29x10^{6}$ $\pm 8.51x10^{4}$ in PBS-treated animals to $1.40x10^{5}$ $\pm 1.49x10^{4}$ when assessed after four injections of toxin.

DTx administration to naïve CD11c.iDTR mice led to a significant ablation of all cDC subsets, with remaining $CD11c^{hi}MHCII^{hi}$ cells almost entirely restricted to the DN subset (**4.5E**). $CD4^{+}$ cDCs decreased in number from $5.72x10^{5}$ $\pm 5.60x10^{4}$ in PBS treated, naïve animals to $9.85x10^{3}$ $\pm 5.30x10^{2}$ ($p<0.001$) after DTx treatment (**4.5F**).

CD8α^+ cDCs were depleted to a similar efficiency, decreasing from $1.47x10^5$ $\pm1.19x10^4$ to $1.43x10^3$ $\pm5.29x10^2$ ($p<0.001$) after administration of DTx. Although highly significant, depletion of DN cDCs was less effective, with 1.20 $x10^5$ $\pm1.32x10^4$ cells from this subset remaining in DTx treated mice, compared with $5.01x10^5$ $\pm3.15x10^4$ cells ($p<0.001$) in PBS-treated control animals. CD11c.iDTR mice should therefore provide an effective tool to address the impact of conventional cDC subset depletion in the chronic stages of infection with *Leishmania donovani*.

4.2.6 Effects of DTx administration on spleen cell composition in CD11c.iDTR mice

Although administration of DTx should have a limited effect on non-CD11c expressing cells, evidence for promiscuous activity of the CD11c promoter required an assessment of the impact of DTx administration on other splenic immune cell compartments. The frequencies (**4.6A**) and numbers (**4.6B**) of splenic CD3ε^+ T cells and B220$^+$ B cells were unaffected by administration of DTx, with T cells at a frequency of 27.75 ±0.87% and number of $6.02x10^7$ $\pm3.29x10^6$ in PBS-treated CD11c.iDTR mice, in contrast to 26.46 ±2.13% and $4.44x10^7$ $\pm5.61x10^6$ in spleens after DTx administration. B220$^+$ cells represented 57.80 ±1.12% of splenocytes in PBS-treated mice, representing a total of $2.88x10^7$ $\pm1.53x10^5$ cells with this phenotype. Injection of DTx did not significantly alter the frequency or number of these cells, with spleens of treated mice containing $2.03x10^7$ $\pm2.66x10^6$ B220$^+$ cells, making up 57.70 ±1.17% of splenocytes after DTx injection.

$CD4^+$ and $CD8\alpha^+$ T cell frequency (**4.6C**) and number (**4.6D**) were similarly unaffected by DTx administration, with only slight, but non-significant, reductions in splenic T cell numbers - probably as a result of decreases in total spleen cellularity. Frequencies of splenic $CD4^+$ T cells were equivalent in PBS and DTx treated mice, at 16.02 ±0.40 and 15.31 ±0.84%, respectively. Numbers of these cells were also equivalent in both groups, at $4.61x10^6$ $±8.62x10^4$ and $3.17x10^6$ $±6.14x10^5$ in PBS and DTx treated mice. Administration of DTx led to a slight decrease in the frequency of $NK1.1^+CD11b^+$ NK cells from 5.04 ±0.07% in PBS treated mice to 4.33 ±0.36% after DTx injection (**4.6E**). This was reflected by a significant decrease in the number of these cells, dropping from $5.24x10^6$ $±1.92x10^5$ in PBS treated animals to $3.31x10^6$ $±4.67x10^5$ ($p<0.05$) after DTx treatment (**4.6F**). A slight, but insignificant, decrease in the frequency of splenic $NK1.1^+$ cells, from 1.91 ±0.15 to 1.74 ±0.23% was also seen after DTx administration, represented by a drop in the number of these cells from $1.97x10^6$ $±1.21x10^5$ in spleens of PBS treated mice to $1.29x10^6$ $±8.25x10^4$ after injection of the toxin.

The frequency (**4.6G**) and number (**4.6H**) of $Gr\text{-}1^{hi}$ cells in CD11c.iDTR mice treated with DTx decreased to 1.83 ±0.14% of splenocytes, or $1.40x10^6$ $±1.92x10^5$ cells, from 3.15 ±0.20% and $3.26x10^6$ $±1.04x10^5$ cells (p=ns) in PBS treated animals. This was accompanied by a reciprocal increase in the frequency of $Gr\text{-}1^{hi}CD11b^{hi}$ cells, from 3.88 ±0.28% to 10.09 ±1.49% ($p<0.05$) of splenocytes and in cell number from $4.04x10^6$ $±3.16x10^5$ to 7.73 $±1.45x10^6$ ($p<0.05$) after DTx administration. Therefore, administration of four doses of DTx to naïve CD11c.iDTR mice led to a significant reduction in the number of splenic $NK1.1^+CD11b^+$ cells, in addition to a significant expansion in the number of $Gr\text{-}1^{hi}CD11b^{hi}$ neutrophils in the spleen.

4.2.7 Conditional ablation of CD11c^{+} cells during chronic infection reduces splenic pathology, enhances nitric oxide production and initiates parasite clearance

In order to assess the impact of CD11c-depletion on parameters of disease, CD11c.iDTR mice infected with *L. donovani*, or age-matched naïve controls, were treated with PBS or 4ng/g DTx every 48 hours from day 21 of infection (**4.7A**). At day 28 mice were killed and effects on spleen cell composition determined by flow cytometry. The effects of DTx administration to chronically infected mice were broadly equivalent to those seen after DTx administration to naïve CD11c.iDTR animals (data not shown, cf. **4.6**). The frequencies of splenic Gr-1^{hi} cells were equivalent, at 0.74 ±0.07% vs 0.94 ±0.02%, in PBS and DTx treated mice respectively (**4.7B**), with Gr-1^{hi}CD11b^{hi} cells increasing significantly in frequency from 1.46 ±0.18% to 2.38 ±0.29% ($p<0.05$) after DTx administration. In contrast, treating infected CD11c.iDTR mice with DTx led to a decrease in the number of Gr-1^{hi} cells from 5.59x10^{6} ±5.10x10^{5} to 3.04 x10^{6} ±3.42x10^{5} after treatment (**4.7C**). This was accompanied by a slight decrease in the number of Gr-1^{hi}CD11b^{hi} cells from 1.15x10^{7} ±2.22x10^{6} in PBS treated mice to 7.33x10^{6} ±5.26x10^{5} after DTx administration.

Administration of DTx to chronically infected CD11c.iDTR mice also allowed for the efficient ablation of splenic CD11c-expressing cells (**4.7D-F**). This was represented by depletion levels in infected mice across two pooled experiments (n=8) of 31.22 ±4.74% for CD11c^{int}MHCII^{-} cells, 52.99 ±5.00% for CD11c^{int}MHCII^{hi} cells and 78.39 ±5.07% from CD11c^{hi}MHCII^{hi} cells (**4.7E**). As in naïve CD11c.iDTR mice treated with DTx, cDC subsets were also depleted to high efficiency in infected mice, with 98.23 ±0.25%, 75.56 ±3.88% and 99.08 ±0.30% of CD4^{+}, DN and CD8α^{+} cDC subsets depleted respectively, after DTx administration (**4.7F**).

Depletion of $CD11c^+$ cells in chronically infected CD11c.iDTR mice had a profound impact on splenic pathology, reflected by dramatically reduced spleen size in these animals, from 3.94 ±0.22% of total body weight in PBS-treated, infected CD11c.iDTR mice, to 2.14 ±0.14% ($p<0.0001$) of body weight in DTx treated animals (**4.7G**). Treatment of naïve mice with DTx had no impact on spleen size, with spleens of naïve, PBS treated animals at 0.35 ±0.02% of body weight compared to 0.37 ±0.04% of body weight after injection of DTx. Reduced splenic pathology was accompanied by significantly enhanced production of Nitric Oxide by adherent splenocytes, with NO_2- levels in culture supernatant increasing from 23.04 ±2.51 μM in PBS-treated, infected mice to 92.36 ±21.89 μM ($p<0.05$) after culture of adherent splenocytes from DTx-treated animals (**4.7H**). NO_2- was not detectable in culture supernatants of adherent splenocytes isolated from naïve animals treated with either PBS or DTx. Ablation of $CD11c^+$ cells also had a significant effect on splenic parasite burden, represented by a decrease in LDUs from 101.54 ±23.02 in PBS-treated animals to 26.98 ±9.45 ($p<0.05$) after ablation of $CD11c^+$ cells (**4.7I**). Levels of circulating IL-27 were dramatically enhanced as a result of infection, with PBS-treated CD11c.iDTR serum containing 287.45 ±23.05pg/ml of IL-27p28 protein, up from a baseline of 5.85 ±1.15pg/ml ($p<0.0001$) in naïve PBS-treated animals (**4.7J**). Levels of serum IL-27p28 were similarly elevated in DTx-treated infected mice, but this was not significantly changed from PBS-treated animals at 336.96 ±20.68 pg/ml.

4.2.8 Effects of DTx administration on splenic architecture in naïve and chronically infected CD11c.iDTR mice

In order to determine any impact of DTx administration on the splenic architecture of naïve and infected CD11c.iDTR mice, cryosections of splenic tissue were assessed by

confocal microscopy for expression of CD169, CD3ε and B220 (**4.8A**). DTx administration to naïve CD11c.iDTR mice had a minor effect on populations of $CD169^+$ marginal zone macrophages located in the marginal zone, whilst in infected mice, DTx treatment resulted in a significant ablation of these cells.

4.2.9 Ablation of $CD11c^+$ cells during chronic infection impairs the generation of $IL\text{-}10^+$ and $IFN\gamma^+IL\text{-}10^+$ antigen-specific $CD4^+$ T cells *in vivo*

To further examine the mechanisms behind the dramatic improvement in pathology and disease progression after $CD11c^+$ cell ablation, splenocytes from naïve and infected PBS and DTx-treated animals were assessed for antigen-specific cytokine producing capacity (**4.9**). Using *L. donovani*-pulsed BMDCs as a source of antigen during restimulation, intracellular cytokine staining revealed populations of splenic $CD3\varepsilon^+CD4^+$ T cells capable of producing IFNγ, IL-10 or both, with responding cells restricted to splenocytes isolated from infected hosts (**4.9A**). Ablation of $CD11c^+$ cells did not affect the frequency of antigen-specific $IFN\gamma^+$ T cells (35.78 ±4.18% in PBS-treated mice vs 30.33 ±5.32% after DTx administration; **4.9B**). Splenocytes from naïve mice did not respond above the background of the assay. The frequency of antigen specific $CD3\varepsilon^+CD4^+$ T cells capable of IL-10 production decreased as a result of $CD11c^+$ cell depletion, from 0.93 ±0.28% to 0.39 ±0.07% (p=0.07, ns) (**4.9C**). Antigen specific $CD4^+$ T cells capable of the simultaneous production of IFNγ and IL-10 were significantly affected by depletion of CD11c-expressing cells, reduced by 50% in frequency from 2.62 ±0.31% to 1.28 ±0.11% (p=0.0009) of viable $CD4^+$ T cells after administration of DTx to infected CD11c.iDTR animals (**4.9D**). Therefore, depletion of $CD11c^+$ cells during chronic infection impairs the generation, survival, maintenance or recruitment of antigen-specific $CD3\varepsilon^+CD4^+$ T cells capable of IL-10 production, either

alone or in conjunction with IFNγ, but leaves effector cytokine production by these cells largely intact. Administration of DTx to chronically infected, *Cre*$^{-ve}$ littermates failed to ablate splenic DCs and had no discernable impact upon any of the parameters discussed above (data not shown).

4.2.10 Neutrophil influx due to DTx administration does not account for the altered CD4$^+$ T cell phenotype after ablation of CD11c$^+$ cells

As administration of DTx to naïve or infected CD11c.iDTR mice led to an increase in neutrophils in the spleen, it was important to determine whether enhanced neutrophil numbers contributed to the altered CD4$^+$ T cell phenotype observed as a result of CD11c$^+$ cell ablation. 1A8 is an antibody that recognises Ly6G [513] and enables the specific depletion of neutrophils *in vivo.* Neutrophil depletion was carried out in chronically infected CD11c.iDTR mice having received DTx, in addition to chronically infected C57BL/6 mice in order to control for any effects of neutrophil depletion on CD4$^+$ T cell cytokine phenotype in CD11c-sufficient animals. Mice received 1A8 or 2A3, an isotype control antibody, intraperitoneally at days 20, 23 and 26 post-infection.

Administration of 1A8 to chronically infected CD11c.iDTR or C57BL/6 mice resulted in the efficient depletion of CD11b$^+$Ly6G$^+$ neutrophils, with limited or no depletion of CD11b$^+$Ly6C$^+$ cells (**4.10**). This was reflected by a highly significant decrease in the frequency of neutrophils from 11.55 ±1.05% of CD11b$^+$ cells in CD11c.iDTR mice receiving 2A3 to 3.02 ±0.64% ($p<0.001$) in animals after 1A8 administration (**4.10A&C**). CD11b$^+$Ly6C$^+$ cells were consequently increased in frequency, from 14.02 ±1.66 to 25.52 ±0.87% as a result of 1A8 injection ($p<0.001$). Levels of CD11c$^+$ cell

ablation were consistent with that observed in the absence of either antibody, as well as between the 2A3 and 1A8-treated groups (data not shown). Depletion of $CD11b^+Ly6G^+$ neutrophils was also evident after 1A8 administration to infected C57BL/6 mice, with these cells decreasing significantly in frequency from 14.80 ±3.27 to 1.47 ±0.54% ($p<0.05$) after treatment with 1A8. Similar to CD11c.iDTR mice, the decrease in neutrophil frequency was associated with a concomitant increase in the frequency of $CD11b^+Ly6C^+$ cells, from 12.03 ±1.54 to 27.64 ±2.49% ($p<0.01$) after 1A8 administration.

In order to address any impact of neutrophil influx upon the altered CD4+ T cell phenotype observed after depletion of $CD11c^+$ cells, $CD3\varepsilon^+CD4^+$ T cells from DTx-treated infected CD11c.iDTR mice, having received either 2A3 or 1A8, were restimulated with PMA and Ionomycin and assessed by flow cytometry for expression of IFNγ and IL-10 (**4.10E&G**). Levels of cytokine production were broadly equivalent in infected, $CD11c^+$ cell-depleted mice in the presence or absence of neutrophils. This was the case for both effector and regulatory cytokine production, with $IFN\gamma^+$ T cells at 43.68 ±1.17 and 35.12 ±2.22%, IL-10^+ T cells at 0.94 ±0.06 and 0.98 ±0.16% and $IFN\gamma^+$IL-10^+ T cells at 1.69 ±0.37 and 1.89 ±0.40% after 2A3 or 1A8 administration, respectively.

In order to confirm whether neutrophil depletion in itself impacted upon $CD4^+$ T cell polarisation during chronic infection, $CD3\varepsilon^+CD4^+$ T cells from chronically infected C57BL/6 mice treated with 2A3 or 1A8 were assessed for cytokine production after restimulation in the same way. Ablation of neutrophils had a similarly limited effect on T cell phenotype in mice with an intact $CD11c^+$ compartment, with $IFN\gamma^+$ T cells at

36.74 ±16.43 and 41.01 ±18.34%, IL-10^+ T cells at 0.51 ±0.23 and 0.50 ±0.22% and IFNγ^+IL-10^+ T cells at 3.94 ±1.76 and 5.21 ±2.33% after treatment with 2A3 or 1A8, respectively. Therefore the reduced frequency of IFNγ^+IL-10^+ T cells in chronically infected CD11c.iDTR mice after ablation of $CD11c^+$ cells is unlikely to be as a consequence of the splenic neutrophil influx associated with DTx administration.

4.2.11 Adoptive transfer of $CD11c^+$ cells from chronically-infected mice confirms their key contribution to the maintenance of chronic disease

The previous ablation studies had suggested a role for $CD11c^+$ cells, including $CD11c^{hi}$ cDCs, in the establishment of chronic infection in the spleen. Although leading to the depletion of ~80% of $CD11c^{hi}MHCII^{hi}$ cDCs, DTx administration to infected CD11c.iDTR mice also led to ablation of other CD11c-expressing populations, albeit at lower levels, thus making an association between chronic infection and a particular CD11c-expressing cell type difficult. Therefore, CD11c ablation and subsequent adoptive transfer approaches were used to assess the relative contribution of $CD11c^{hi}$ cDCs and $CD11c^{int}$ cells to the establishment of chronic disease (**4.11**).

Groups of CD11c.iDTR mice were infected with *Leishmania donovani* and disease allowed to progress for 21 days. At day 21 of infection, mice received PBS or DTx, with some DTx-treated groups also receiving splenic $CD11c^{hi}MHCII^{hi}$ cDCs and/or $CD11c^{int}$ cells sorted from day 21-infected $CD45.1^+$ congenic wildtype donors. $CD11c^+$ cell transfers were performed ~12hrs after initial DTx treatment. DTx administration on days 23, 25 and 27 was then performed as previously, before the end of the experiment at day 28 (**4.11A**). Transferred cell populations were sorted to high purity, with $CD11c^{hi}$

cDCs at >98% and $CD11c^{int}$ cells at >97% purity when isolated from infected B6J.CD45.1 mice at day 21 of infection (**4.11B**).

As the transferred cells were from congenic $CD45.1^{+}$ hosts, it was possible to track them after injection by flow cytometry (**4.11C**). However when assessed, the few cells remaining in the $CD11c^{hi}MHCII^{hi}$ gate after 4 doses of DTx were exclusively $CD45.2^{+}$ and at frequencies consistent with that previously observed after CD11c ablation. $CD11c^{int}$ cells were also entirely derived from the endogenous $CD45.2^{+}$ population, suggesting that very few of the transferred cells, of either population, remained or had engrafted 7 days after transfer.

Despite the lack of detectable transferred cells at day 28, transfer of $CD11c^{+}$ cells from chronically infected mice into infected, CD11c-depleted animals substantially altered the splenic response to *Leishmania donovani* (**4.11D-F**). Whereas ablation of $CD11c^{+}$ cells reduced spleen size from 2.92 ±0.53% of body weight in CD11c-sufficient mice to 1.11 ±0.09% after DTx administration ($p<0.05$), adoptive transfer of CD11c-expressing cells from infected mice significantly restored splenomegaly. One week post-transfer, spleen size was 2.47 ±0.24%, 2.44 ±0.24% and 2.29 ±0.16% (all $p<0.01$) in CD11c-depleted mice receiving $CD11c^{hi}$, $CD11c^{int}$ or both populations of cells, respectively (**4.11D**). Therefore, all $CD11c^{+}$ cell populations restored splenomegaly after adoptive transfer to CD11c-depleted mice.

Alongside the substantial effects on splenomegaly, CD11c ablation and subsequent adoptive transfer also impacted upon splenic parasite burden (**4.11E**). Consistent with

previous observations (**4.7**), ablation of $CD11c^+$ cells during chronic infection led to a significant decrease in splenic parasite burden (116.39 ±24.15 vs 42.52 ±12.21 LDUs in PBS treated vs DTx treated mice, respectively; $p<0.05$). Transfer of $CD11c^{hi}$ cells from infected mice significantly restored the splenic parasite burden (115.11 ±25.20 LDUs; $p<0.05$). Although not significantly so, $CD11c^{int}$ and mixed $CD11c^{hi}$/$CD11c^{int}$ transfers showed a similar trend and increased parasite burden in the spleen.

Ablation and subsequent transfer of $CD11c^+$ cells during infection also had an impact on the systemic response to *L. donovani* (**4.11F**). Although not significantly altered, ablation of $CD11c^+$ cells led to a trend toward decreased levels of IL-10 in sera from infected CD11c.iDTR mice. However, transfer of $CD11c^+$ cells from infected mice significantly augmented levels of serum IL-10, with transfer of $CD11c^{hi}$ cDCs increasing levels of IL-10 to 64.50 ±12.23 pg/ml ($p<0.05$), $CD11c^{int}$ cells to 58.80 ±7.53 pg/ml ($p<0.01$) and both populations to 40.00 ±5.03 pg/ml ($p<0.05$) 7 days after transfer. Therefore, transfer of $CD11c^+$ cells significantly enhanced systemic levels of IL-10 after CD11c depletion.

4.2.12 $CD11c^{hi}$ cDCs are the major cell type facilitating expansion of splenic $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells during chronic infection *in vivo*.

The previous data supported a role for CD11c-expressing cells in the maintenance of chronic infection. However, it appeared that $CD11c^{hi}$ cDCs and $CD11c^{int}$ cells both had the capacity to mediate these pathological processes. To further investigate any differences in the function of these cell populations during infection, $CD3\varepsilon^+CD4^+$ cells

from each experimental group were assessed by flow cytometry for expression of IFNγ and IL-10 after restimulation with PMA & Ionomycin (**4.12**).

Ablation of $CD11c^+$ cells during infection and the subsequent adoptive transfer of CD11c-expressing cells from chronically infected mice altered the cytokine producing capacity of $CD4^+$ T cells during infection (**4.12A**). $CD4^+$ T cells producing IFNγ alone were not significantly affected by CD11c depletion. However, the adoptive transfer of $CD11c^{hi}$, $CD11c^{int}$ or both populations of cells significantly increased the frequency of $CD4^+IFN\gamma^+$ cells to 27.42 ±1.92, 27.16 ±1.92 and 23.54 ±0.75% (all $p<0.01$), respectively, by day 28 of infection (**4.12B**). There were no differences in the capacity for either cell population to affect IFNγ-producing T cell frequencies. $IL\text{-}10^+$ T cells were slightly reduced in frequency by CD11c ablation, from 2.74 ±0.55 to 2.01 ±0.31% after DTx administration (**4.12C**). Adoptive transfer of either population into CD11c-depleted animals had little effect on the polarisation of IL-10-producing T cells, reflected in frequencies of 2.05 ±0.15, 2.39 ±0.10 and 2.11 ±0.08% after transfer of $CD11c^{hi}$, $CD11c^{int}$ or both populations.

In contrast to the relatively modest changes in IFNγ and IL-10 single producing cells, $CD4^+$ T cells capable of simultaneous IFNγ and IL-10 production were substantially affected by CD11c ablation and subsequent adoptive transfer of CD11c-expressing cells during infection. $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells decreased in frequency from 8.21 ±1.97 to 2.76 ±0.87% after DTx administration, with this population being substantially restored after adoptive transfer of $CD11c^{hi}$ cells, to 7.21 ±0.59% ($p<0.01$). In contrast, adoptive transfer of $CD11c^{int}$ or both populations of cells only slightly increased the frequency of

splenic $CD4^+$ T cells with this phenotype, at 4.34 ±0.80 and 4.81 ±0.43% after transfer (p=ns), significantly less than that generated after transfer of $CD11c^{hi}$ cells (both $p<0.05$).

Therefore, $CD11c^{hi}MHCII^{hi}$ cells are the major cell type responsible for the generation of $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells as a result of chronic *Leishmania donovani* infection *in vivo*.

4.3 Discussion

The data presented here further elucidate the mechanistic basis for impaired IL-12 production by splenic cDCs during chronic infection by identifying an auto-regulatory loop in which IL-10 and IL-27 production by cDCs modulates their function and thus contributes to their limited capacity for T cell activation *in vitro*. Furthermore, a fundamental role for altered dendritic cell function in the maintenance of chronic infection was established by conditional ablation and subsequent adoptive transfer of these cells *in vivo*, highlighting the paradoxical role that cDCs play by impairing effective immune responses during experimental *Leishmania donovani* infection.

Extending observations on cytokine dysregulation from the previous chapter, cDCs isolated from chronically infected mice showed an impaired capacity to produce IL-12p70 directly *ex vivo* or after LPS stimulation. This is in accordance with previous data indicating that *ex vivo* IL-12p70 production by cDC subsets is completely abolished in day 28-infected BALB/c mice [108]. IL-10 receptor blockade initiated enhanced production of IL-12p70 by cDCs from both naïve and infected mice, but this effect was most pronounced upon cDCs from chronically infected animals. cDCs isolated from chronically infected mice also produced significant amounts of IL-10, levels of which were increased dramatically when IL-10 uptake by cDCs was prevented after culture with an αIL-10R antibody, confirming observations from the previous chapter that suggest autocrine uptake of IL-10 by cDCs occurs as a result of infection.

IL-10 has long been known as a potent suppressor of IL-12 production by APCs [514-516], and a growing body of evidence suggests that autocrine IL-10 signalling in DC

regulates their functional activation, including costimulatory molecule expression and pro-inflammatory cytokine production [517]. Autocrine IL-10 signalling plays a role in mediating the IL-12-inhibiting effects of the chemoattractants MCP1-4 [518], in addition to regulating the differentiation, IL-12 production and T cell polarising capacity of human monocytes [519, 520] and mouse BMDCs [521] *in vitro*. However, IL-12p70 production by DCs can be inhibited independently of autocrine IL-10 signalling [522] and so other mechanisms may also be involved in the cytokine dysregulation observed in this study. Interestingly, suppression of IL-12p70 production by endogenous IL-10 after TLR ligation is more pronounced in murine splenic cDCs than either macrophages or BMDCs [492], suggesting a particular sensitivity of these cells to this process of self-regulation.

Although only capable of producing very little IL-10, preventing IL-10 uptake by naïve cDCs in culture also significantly increased their capacity for IL-12p70 secretion, indicating that IL-10 is highly efficient at regulating pro-inflammatory cytokine production by cDCs, even at low levels. This process is thought to be mediated by Stat3 activation, as ablation of Stat3 specifically in cDCs leads to enhanced IL-12 production and resistance to inhibition of DC activation by IL-10 *in vivo* [523], with suppressor of cytokine signalling (SOCS)-3 also playing a role *in vitro* [524]. An additional mechanism by which autocrine IL-10 signalling in DCs impairs IL-12 production through Mammalian target of rapamycin (mTOR) has also been proposed [217], so further work will be required to elucidate the molecular mechanisms underlying the cytokine dysregulation described in this study. Of relevance to the enhanced expression of TLR2 on cDCs during infection described in Chapter 3, in addition to the potential recognition of *Leishmania* spp by this receptor [338, 342, 481-483, 485], TLR2 signalling has been shown to induce IL-10 and result in the autocrine suppression of IL-

12p35 mRNA in human DCs [525], suggesting a potential link between TLR2 signalling and the suppression of IL-12 production by autocrine IL-10 in cDCs identified here.

Studies identifying such an autocrine regulatory network during infection are more limited, but preventing endogenous IL-10 production by DCs initiates more effective Th1 responses after vaccination against *Chlamydia* infection [526], autocrine IL-10 signalling prevents IL-12 production by BMDCs in response to BCG infection *in vitro* [491] and human DC production of IL-12p70 is prevented as a result of autocrine IL-10 signalling after *Neisseria meningitidis* infection *in vitro* [527]. Furthermore, autocrine IL-10 production by splenic DCs in response to yeast zymosan inhibits pro-inflammatory cytokine production *in vitro* [209] and IL-10 production by innate cells inhibits DC-derived IL-12 production after vaccination against *L. major* [528], suggesting that autocrine regulation of IL-12 production also occurs in other *in vivo* systems. In addition, endogenous IL-10 attenuates BMDC maturation and IL-12 production in response to *Streptococcus pneumoniae*, as well as limiting apoptosis of these cells after activation [529]. However, autocrine IL-10 signalling has also been shown to inhibit expression of Bcl-2 family members in BMDCs, thus increasing their apoptotic potential [530], indicating that further work is required to address whether autocrine IL-10 may also impact upon splenic cDC lifespan as a result of infection *in vivo*.

Despite the beneficial effect of combined IL-10R blockade and IL-27p28 neutralisation on *in vitro* Th1 polarisation by cDCs from infected mice described in Chapter 3, neutralisation of IL-27 during *ex vivo* cDC culture had a limited impact on cytokine

production by these cells. As cDCs from naïve mice produced very little IL-27 *ex vivo*, it was unsurprising that neutralisation of this cytokine had no effect on IL-12p70 production by these cells, alone, in conjunction with αIL-10R, or in the presence of LPS. However, culture with αIL-27p28 had a similarly limited impact on IL-12p70 production by cDCs which were previously determined as significant producers of elevated levels of IL-27 when isolated during chronic infection. This was unexpected, as rIL-27 has been reported to suppress IL-12 production by macrophages *in vitro* [496, 531] and BMDCs generated from WSX-1 (IL-27Rα)$^{-/-}$ mice show enhanced production of IL-12p40 and p70, as well as augmented costimulatory molecule expression, in response to TLR ligation *in vitro* [495].

It is possible that the autocrine suppression of IL-12p70 production by IL-10 overrides any inhibitory effect of IL-27 on IL-12 production; however, combined inhibition of IL-10 and IL-27 signalling did not result in an additive effect on the rescue of IL-12p70 production by cDCs, indicating a limited role for IL-27 in suppressing IL-12p70 in this situation. Neutralisation of IL-27 actually led to slightly enhanced IL-10 production by cDCs after LPS stimulation, indicating a potential negative regulation of IL-10 expression by IL-27- an effect reported in human monocytes [532]. If this is indeed occurring, then the enhanced IL-10 production by cDCs in the presence of αIL-27p28 alone may explain the limited capacity for neutralisation of IL-27 to enhance IL-12 production by cDCs; however, a recent study showing that IL-27 production by macrophages is required for optimum IL-10 production suggests that the opposite may be the case [533]. Further work will be required to more fully elucidate the mechanisms, but the data presented here indicate that autocrine IL-10 signalling plays a dominant

role in modulating pro-inflammatory cDC cytokine production during chronic *L. donovani* infection.

As there appeared to be a limited role for IL-27 in directly regulating cytokine production by cDCs, the impact of exogenous IL-27 on the polarisation of OTII.*Rag*2$^{-/-}$ TCR transgenic T cells was assessed *in vitro*. As previously discussed, IL-27 has a documented role in the generation of IFNγ^{+}IL-10^{+} CD4^{+} T cells. Despite this, five day cultures with cDCs in the presence of exogenous recombinant IL-27 did not lead to IL-10 production by OTII T cells after restimulation with PMA and Ionomycin. Instead the contribution of IL-27 to the negative regulation of IL-12-induced Th1 polarisation, partially dependent upon APC-derived IL-10, was apparent.

As discussed in Chapter 3, such a lack of IL-10 production by OTII T cells after culture with IL-27 could be due to variations in the *in vitro* systems used for assessing its function. This study utilised recombinant IL-27 at a concentration shown to induce IFNγ^{+}IL-10^{+} cells from draining LN of mice infected with *L. major* [486], suggesting that the availability of IL-27 is unlikely to be a reason for the lack of CD4^{+}IL-10^{+} T cells generated after *in vitro* culture. Other components of the culture system may however be playing a role; the vast majority of previous studies describing IL-27's role in T cell IL-10 production utilised a much 'stronger' stimuli of αCD28 and/or αCD3, in many cases in an APC-free system, than the cDCs plus PMA and Ionomycin used here [254, 255, 259, 260, 262]. However the part played by IL-27 in the polarisation of CD4^{+} T cells to an IFNγ^{+}IL-10^{+} phenotype has been reported in a system involving CD11c^{+} APCs in conjunction with PMA and Ionomycin restimulation [486], so there is some

evidence that both culture / restimulation methods are permissive to the generation of T cells with this phenotype.

Previous studies showing co-expression of IL-10 and IFNγ by T cells *in vitro*, irrespective of whether the role of IL-27 was addressed, have been restricted to using non-TCR transgenic T cells [486, 534], T cells bearing auto-antigen specific TCRs [254] or an OVA-specific DO11.10 TCR from mice on a BALB/c background [262, 488]. Therefore it is possible that the nature of the TCR transgenic T cell system used in this study had specific differences which impacted upon the potential for $IFN\gamma^{+}IL\text{-}10^{+}$ T cell generation by cDCs *in vitro*. Further work will be required to determine if the lack of any co-expression of IFNγ and IL-10 in this study was due to an intrinsic incapacity of OTII.*Rag*$2^{-/-}$ T cells to acquire this phenotype after stimulation.

Interestingly, IL-27 partially inhibited the IL-12-induced Th1 polarisation of OTII T cells after culture. A capacity for IL-27 to affect Th1 polarisation has been reported in a wide range of relevant experimental infections. Mice deficient in WSX-1 and thus unable to respond to IL-27 show exacerbated Th1 responses, pro-inflammatory cytokine production and severe liver pathology when infected with *Leishmania donovani* [535], *Plasmodium berghei* [536], *Trypanosoma cruzi* [239] or *Toxoplasma gondii* [238]. A very similar pathology is observed in WSX-$1^{-/-}$ mice infected with *Mycobacterium tuberculosis*, where exacerbated IFNγ production and T cell activation leads to enhanced macrophage activity and bacterial clearance, but severe immunopathology and early death [496]. However, IL-27R deficiency does not affect IFNγ production by T cells in mice infected with *Listeria monocytogenes* [262], indicating that the effects of

IL-27 signalling on IFNγ^{+} T cell polarisation may be dependent upon the nature of infection.

Such a dramatic impact of IL-27 on IFNγ-production by CD4^{+} T cells may be due to mechanisms independent of IL-10, as serum and splenocyte production of IL-10 is equivalent to that of wildtype animals at day 30 during *L. donovani* infection of WSX-1$^{-/-}$ mice [535]; IL-10 levels are equivalent in liver mononuclear cells from wildtype and WSX-1$^{-/-}$ mice infected with *T. cruzi* [239]; and administration of recombinant IL-10 to *P. berghei* infected IL-27R-deficient mice is unable to prevent immunopathology [536]. Although IL-12 expression is enhanced at the mRNA and protein level during infections of WSX-1$^{-/-}$ mice, [496, 535, 536], no study has addressed whether DCs are the source of enhanced IL-12; assays in previous studies were performed on whole tissue or serum. However the exacerbated T cell IFNγ production during *T. gondii* infection of WSX-1$^{-/-}$ mice is thought to be independent of the regulation of IL-12 in DCs [238], therefore suggesting that IL-27 can modulate T cell IFNγ production independently of DC-derived IL-10 and IL-12, consistent with the observations that IL-27 blockade had little effect on production of these cytokines by cDCs in this study.

Perhaps the most convincing evidence of a role for IL-27 in directly regulating IL-12-induced Th1 induction, similar to that described here, comes from recent work describing the function of liver myeloid DCs (mDCs) [537]. These cells preferentially express IL-10 and IL-27 after activation, have a higher threshold for LPS stimulation and a reduced capacity for T cell polarisation, in part due to their low levels of IL-12 production. Crucially, culture of liver mDCs with wildtype or WSX-1$^{-/-}$ T cells in the presence of exogenous IL-12 revealed significantly augmented IFNγ production from T

cells which could not respond to the IL-27 produced by mDCs, strongly indicating that IL-27 is able to regulate Th1 polarisation induced by IL-12 *in vitro*.

As these cells co-express IL-10, similar to the splenic cDCs described in this study, it is not possible to segregate the respective roles for IL-10 and IL-27 in this process. However, the data presented in this study indicates that the inhibition of IL-12-induced IFNγ from OTII T cells by IL-27 is at least partially dependent upon IL-10, as cultures utilising B6.*Il10*$^{-/-}$ cDCs as APCs revealed a more limited capacity for IL-27 to affect Th1 polarisation when compared to IL-10-sufficient cDCs. It is unclear whether this is due to a requirement for synergy between IL-10 and IL-27 to fully suppress Th1 polarisation, but as IL-27 has been recently reported to modulate other effector functions of IL-12 [538] and antagonise gp130-mediated pro-inflammatory cytokine signalling [539], further work is warranted to elucidate the mechanism behind this observation. Nevertheless, the regulation of IL-12 function by IL-27 suggested by this data allows the creation of a model by which autocrine IL-10 signalling as a result of chronic infection limits IL-12 production by cDCs, with the effector function of the low levels of IL-12 remaining being modulated by IL-27 produced by the same cells (cf. Chapter 6). This goes some way to explaining the observed synergy between IL-10R blockade and IL-27p28 neutralisation in improving the Th1 polarising capacity of cDCs described in the previous chapter.

In summary, the data presented here are the first evidence that the profound suppression of IL-12 production by splenic cDCs as a result of *L. donovani* infection *in vivo* is, at least in part, mediated by autocrine uptake of IL-10. In addition, IL-27 contributes to the impaired T cell polarising capacity of these cells by modulating IL-12-induced IFNγ

production by $CD4^+$ T cells *in vitro*. As both T cells and APCs express the IL-27 receptor [218, 540, 541], determining whether sensitivity to IL-27 and/or IL-10 is required by cDCs and/or T cells is necessary. To this end, the use of cell-specific *Cre* recombinase-expressing mice, crossed with strains bearing a *loxP*-flanked WSX-1 or IL-10R gene, would enable the relative importance of these cytokines to the progression of disease to be addressed. Further work will also be required to elucidate the signalling pathways responsible for this novel auto-regulatory network, in addition to confirming the existence of such a pathway during *Leishmania donovani* infection in man.

In the absence of tools allowing the specific ablation or *in vivo* neutralisation of IL-27 or the blockade of the IL-27 receptor, an alternative approach was necessary in order to address the function of IL-27 during disease progression. As several members of the IRF family are required for optimal IL-27 expression [497-501], it was hypothesised that mice deficient in IRF7 may have an impaired capacity for IL-27 production similar to other IRF-deficient strains, thus providing a useful way to address the role of IL-27 in chronic *Leishmania donovani* infection *in vivo*.

A lack of IRF7 did not affect splenic pathology or parasite burden by day 28 of infection, in contrast to the higher parasite burden in the marginal zone of IRF7-deficient mice during acute infection [502], and the impaired clearance of parasites from the liver of B6.*Irf7*$^{-/-}$ mice during chronic infection (Beattie *et al*, in press). No study has directly addressed any role for IRF7 in other related parasitic infections, however, mice lacking IRF1, IRF2 or IRF4 all show exacerbated lesion development and impaired parasite clearance after infection with *Leishmania major* [542-545]. Therefore it would seem that the protective role of IRF7 in the response to *Leishmania donovani* is organ

and disease stage-dependent, as it does not appear to contribute to the regulation of splenic pathology during chronic infection.

Chronic infection led to enhanced expression of CD40 and more muted increases in CD86 expression by cDCs, consistent with that described in Chapter 3 and on splenic cDCs during acute infection of BALB/c mice [108]. A lack of IRF7 did not substantially alter this activation, with comparable responses occurring in the presence or absence of IRF7, similar to the comparable levels of CD86 expression by splenic cDCs from wildtype or IRF7-deficient mice infected with murine cytomegalovirus (MCMV) [546]. Splenic immune cell development was normal in B6.*Irf7*$^{-/-}$ mice (cf. Chapter 5), consistent with the normal splenic architecture and liver immune cell composition previously described ([502] and Beattie *et al*). IRF7-deficiency did not affect production of IL-27 during infection, with only a slight reduction in *ex vivo* IL-27 production by cDCs from chronically infected B6.*Irf7*$^{-/-}$ mice compared to cDCs from wildtype animals. This provides the first evidence for intact production of IL-27 by splenic cDCs lacking IRF7 in response to an infection *in vivo* and extends recent observations that *Irf7*$^{-/-}$ BMDCs show normal IL-27 production in response to LPS *in vitro* [500]. Confirming the lack of a requirement for IRF7 in IL-27 production more generally, IL-27p28 was at similar highly elevated levels in sera from C57BL/6 and B6.*Irf7*$^{-/-}$ mice, revealing for the first time that systemic IL-27 is detectable during chronic *L. donovani* infection. In addition, IL-27 production by cDCs in response to LPS or IFNα *in vitro* was intact in IRF7-deficient cells, further suggesting that IRF7 is not required for IL-27 expression under a range of conditions. The slightly reduced levels of IL-27 protein in both cDC supernatants and serum from B6.*Irf7*$^{-/-}$ mice may be due to less efficient IL-27 induction, as IRF7 is critical for IFNα induction [547] and

IFNα potentiates IL-27 production by macrophages [497]. More work will be required to determine the signalling pathways leading to IL-27 production by cDCs during infection, but the data here would suggest that IRF7 is not necessarily required.

Although again not directly addressing IRF7, several interactions between *Leishmania* spp and IRFs in APCs have been reported. IL-12p35 expression is induced directly by IRF1 and IRF8 activity after *L. major* infection of human DCs, a process that does not occur during infection with *L. donovani* and is suggested to contribute to the impaired IL-12 production by these cells in response to this parasite species [548]. Amastigotes of *L. donovani* [549] and *L. amazonensis* [400] are also capable of inhibiting IRF8 and/or IRF1 expression, suggesting that IRFs are a target exploited by parasites in order to establish persistent infection. Indeed, severely impaired IL-12 production has been reported in *L. major* infected *Irf1*$^{-/-}$ [545] and *Irf2*$^{-/-}$ mice [544], underlying the key role for IRFs in effective anti-parasitic immunity. In this study IRF7 deficiency did not affect levels of serum IL-12p70 in chronically infected mice, in contrast to a model of acute MCMV infection where a lack of IRF7 leads to substantially increased serum IL-12p40 and p70 protein levels, as well as increased mRNA encoding IL-12 subunits in splenic tissue [546]. This suggests that IRF7 plays a less important role in IL-12 production during infection than other IRFs such as IRF1 and IRF2 (cf. Chapter 5), although further work will be required to address the exact role of IRF7 in IL-12 production by cDCs as a result of *in vivo* infection; this study only assessed IL-12 in serum, rather than production by defined cell types.

It was important to determine any impact of IRF7-deficiency on the splenic T cell response to *L. donovani,* as previous studies had suggested a role for IRF7 in the

optimal production of IFNγ by hepatic $CD4^+$ T cells during chronic *L. donovani* infection (Beattie *et al*) and splenic $CD8\alpha^+$ T cells during viral infection [547]. In addition, several IRFs are required to generate effective $CD4^+$ T cell responses to *L. major* [542-545, 550, 551], *Toxoplasma gondii* [552] and *Listeria monocytogenes* [553], with T cell IL-10 production also modulated by by IRF1 during *P. berghei* infection [554]. In contrast to viral infection, no defect was observed in the $CD8\alpha^+$ T cell IFNγ response, indicating that the generation of these effector cells during infection is not dependent on IRF7 itself, or IFNα induction. This is in contrast to mice lacking IRF4, which have $CD8\alpha^+$ T cells with severely impaired CTL function and are thus unable to control LCMV infection [555], in addition to IRF8-deficient mice which also show impaired CTL activity during viral infection [556]. Although cytotoxic mediators such as perforin or granzyme B were not directly addressed in this study, the intact levels of IFNγ production detected from *Irf7*$^{-/-}$ $CD8\alpha^+$ T cells suggests that they would have normal functionality, at least in response to an intracellular pathogen.

No significant deficit in IFNγ production by $CD4^+$ T cells was observed at day 28 of infection in *Irf7*$^{-/-}$ mice, although the frequency of $CD4^+IFN\gamma^+$ T cells was slightly reduced in IRF7-deficient animals by this time point. This is in contrast to the impaired hepatic IFNγ response by this stage of infection, where $CD4^+$ T cells capable of IFNγ production are significantly reduced in frequency (Beattie *et al*). Interestingly an enhanced capacity for IL-10 production by $CD4^+$ T cells at day 28 of infection was evident in both the spleen and liver, and may therefore impact upon $CD4^+IFN\gamma^+$ T cell development. Whilst IRF4 is known to directly bind the *Il10* promoter [557] and positively regulate IL-10 expression by $CD4^+$ T cells [558], impaired IL-10 mRNA expression and protein production by splenocytes from *Irf1*$^{-/-}$ mice infected with *T.*

gondii has been reported [559], indicating that IRFs are capable of positive and negative regulation of IL-10 production by immune cells. Further work is required to determine the mechanistic basis behind the enhanced IL-10 production observed by $CD4^+$ T cells lacking IRF7, but unlike the hepatic response (Beattie *et al*) the augmented $CD4^+$ T cell-derived IL-10 levels did not appear to significantly impact upon splenic parasite burden or pathology.

As levels of cDC-derived and serum IL-27 were equivalent in chronically infected wildtype and IRF7-deficient mice, it was perhaps surprising that $CD4^+IL\text{-}10^+$ cells were slightly altered in frequency. However, the differentiation of $CD4^+$ T cells co-producing IFNγ and IL-10 was not impaired by IRF7 deficiency, and it is cells of this phenotype that have been particularly associated with IL-27 for their differentiation [254, 262, 486]. Taken together, it would appear that IRF7 is not required for IL-27 production by splenic cDCs *in vitro* or *in vivo*, has a limited role in effector T cell polarisation in the spleen during chronic parasitic infection and does not substantially impact upon splenic pathology during persistent disease. The reasons underlying the discrepancy between splenic and hepatic requirements for IRF7 will require further investigation.

An integral element of this study was to address the impact of the profoundly altered cDC phenotype upon parameters of chronic disease *in vivo*. To do this a novel mouse system was deployed which enabled the continual ablation of CD11c-expressing cells during ongoing chronic infection with *L. donovani*. The CD11c-DTR strain originally developed by Jung *et al* could only be used for studies requiring transient DC depletion, as toxicity and death was observed 6-7 days after a single injection of DTx [560]. In this study no toxicity was observed in any naïve or infected CD11c.iDTR mice after 4

injections of DTx over a period of 7 days. Although this was an extended period of time, the period of CD11c depletion is not as sustained as the 11 [505, 506] or 14 (Engwerda *et al*, unpublished) days of DTx administration without toxicity previously reported in CD11c-DOG mice, so it is not yet clear whether the CD11c.iDTR strain represents a suitable model for longer-term DC depletion studies.

Levels of cDC depletion were consistently high, with ~90% of splenic $CD11c^{hi}MHCII^{hi}$ cells depleted from naïve animals. However, other CD11c-expressing cells were also depleted, a caveat in the system rarely mentioned or addressed in previous studies. Strategies deployed to overcome this issue will be discussed later in this study. Importantly, the majority of the $CD11c^{hi}$ splenic DC subsets were depleted after DTx administration to infected mice. Subset depletion has not been routinely addressed in previous DC ablation studies, although splenic $CD11b^{+}$ and $CD8\alpha^{+}$ subsets are efficiently depleted in CD11c.DOG mice receiving DTx for 11 days [506]. Although the numbers were significantly reduced, DN cDCs were the only subset partially resistant to DTx administration, with approximately 25% of these cells remaining after DTx treatment. As there is some ambiguity as to the nature of this cDC subset and its relationship to other types of DC elicited during inflammation [47, 63, 64] (cf. Chapter 1), it is possible that cells falling into this gate are replenishing more quickly from a monocyte precursor or express lower levels of CD11c than the other subsets and are thus less susceptible to DTx treatment. Further work will be required to address this, and to also further define any functional alterations in the DCs that remain after depletion.

Activity of the *Itgax*/CD11c promoter is not completely restricted to dendritic cells, with evidence of *Itgax* expression in lineages as distinct as CD8α^+ T cells and plasma cells [561, 562]. As a result of this, administration of DTx to CD11c-DTR mice can lead to the depletion of a range of immune cells, including alveolar macrophages [563], CD169$^+$ lymph node subcapsular sinus macrophages [564], splenic marginal zone and metallophillic macrophages [353, 509, 565], but not F4/80$^+$ red pulp macrophages [353, 506, 509, 565]. In light of this reported promoter promiscuity, it was crucial to determine any effects of DTx administration to CD11c.iDTR mice on cell types other than DCs.

No significant alterations in the frequency or number of T cell subsets or B cells were apparent after ablation of CD11c$^+$ cells. A small, but significant, decrease in NK1.1$^+$CD11b$^+$ cells was observed in spleens of naïve and infected CD11c.iDTR mice after DTx administration, however it is not clear whether this was due to toxin-mediated killing or simply a reflection of the lack of cDC-derived IL-15, previously shown to be essential for NK cell homeostasis and activation in the periphery [566]. There were no other substantial alterations in splenic immune cell compartments that may be attributable to DTx-mediated toxicity, although loss of CD169$^+$ marginal zone macrophages was observed by immunohistochemistry; a similar effect to that observed in previous studies utilising different CD11c ablation systems [351, 565]. However, this technique also revealed the maintenance of F4/80$^+$ red pulp macrophage populations after DTx ablation (not shown), indicating that macrophage populations in CD11c.iDTR mice respond in a similar fashion to other published CD11c-DTR strains.

Administration of DTx to CD11c.iDTR animals did however lead to substantial levels of splenic neutrophilia, particularly in naïve mice. This phenomena has not previously been reported in published studies involving DTx administration i.p., however neutrophilia is observed when $CD11c^{hi}$ cDCs are ablated in a system based upon constitutive expression of the A subunit of DTx under control of the CD11c promoter [567], suggesting that this phenomenon may be due to the presence of the toxin itself. In addition, neutrophils readily phagocytose cells undergoing apoptosis [568] and so the observed influx of neutrophils in the spleen after DTx administration may also be due to DTx-induced death of CD11c-expressing cells. Despite some alterations in other immune cell frequencies, in general the main impacts of DTx administration to CD11c.iDTR mice were restricted to CD11c-expressing cell populations. However strategies to overcome the caveats introduced to the study by the alterations in NK and neutrophil frequency will be addressed later.

Although less efficient than in naïve CD11c.iDTR mice, the strain enabled the effective depletion of $CD11c^{hi}MHCII^{hi}$ cDCs from chronically infected mice with around 80% efficiency. This is similar to the 70-80% efficacy reported for depletion of splenic cDCs during infection with *S. mansoni* [506]. The impact of a relatively short period of CD11c ablation on disease progression was striking. Splenomegaly, a relatively crude indicator of splenic pathology, was dramatically reduced by depletion of CD11c-expressing cells. The reduced level of splenic pathology was similar to that seen after inhibition of vascularisation by Sunitinib, a receptor tyrosine kinase inhibitor (RTKi) [569], as well as parasite killing after drug treatment.

Although not directly addressed in this study, DCs have been reported as potential mediators of angiogenic pathology via production of vascular endothelial growth factor (VEGF); a process induced by IL-10 and Prostoglandin E2 (PGE2) [570]. As IL-10 production by cDCs is apparent from this study and PGE2 is associated with *L. donovani* infection [571, 572], it remains possible that ablation of $CD11c^{+}$ cells reduces levels of IL-10 and PGE2-induced VEGF expression during infection and thus explains the dramatic changes in spleen size observed when these cells were depleted. Furthermore, a population of $CD11b^{+}$ myeloid cells has been reported to control vascular changes at the site of *L. major* infection [573], suggesting the possibility that the other cell types depleted by DTx administration may also contribute to tissue remodelling. However, DCs are also known to produce anti-angiogenic factors such as soluble VEGF-receptor 1 (VEGFR1) after activation [574], so further work will be required to address the capacity for pro-angiogenic factor production by cDCs or $CD11c^{int}$ cell populations as a result of chronic *Leishmania donovani* infection.

Although structural changes were not affected to the same degree as with Sunitinib treatment, there appeared to be some restoration / protection from destruction of splenic architecture; albeit far less striking than that previously reported [569]. Interestingly, Sunitinib is a RTKi with particular affinity for Flt3 and its administration is reported to prevent expansion of cDCs *in vivo* by inhibiting proliferative signals provided by Flt3L in the spleen microenvironment [49, 575]. Therefore it is possible that treatment with Sunitinib may prevent expansion of cDCs, leading to a similar reduction in frequency to that seen after DTx treatment. This could partially explain the similar effects on pathology due to the seemingly disparate interventions of RTKi treatment and CD11c depletion. Further work will be required in order to investigate the potential relationship between $CD11c^{+}$ cells, Sunitinib and tissue remodelling during chronic infection.

As well as the dramatic impact on splenic pathology, ablation of CD11c-expressing cells during chronic infection significantly affected T cell cytokine production, levels of serum IL-10 and the initiation of Nitric Oxide (NO)-dependent parasite clearance. The majority of previous studies assessing the impact of *in vivo* dendritic cell depletion in infectious settings have been restricted to acute stages of infection and thus address the impact of DC ablation on T cell priming, such as the reduced effector responses to acute infection with *L. donovani* [353], *Toxoplasma gondii* [576], HSV-1 [577], *Listeria monocytogenes* and *Plasmodium yoelii* [578], *Streptococcus pyogenes* [579], murine-tropic HTLV-1 [580] and *M. tuberculosis* [581]. As such, this study is one of the first to address the role of DCs in disease pathology by their ablation during ongoing chronic infection.

In this study, depletion of $CD11c^+$ cells after the establishment infection did not significantly affect IFNγ production by $CD4^+$ T cells, with the frequency of these cells only slightly reduced after treatment; suggesting that DCs are not required to maintain the capacity for effector T cell responses after initial priming. This is similar to the effects of DC ablation on $CD4^+$ T cell responses to *M. tuberculosis* where DC are critical for initial priming of $CD4^+$ effector T cell responses, but dispensable for recall Th1 responses after vaccination [581]. However this is in contrast to CD11c-depletion during established infection with *S. mansoni*, where $IFN\gamma^+$ production by $CD4^+$ T cells is significantly reduced by ablation of CD11c-expressing cells [506]; suggesting that in some chronic infections $CD11c^+$ cells are required for maintaining T cell IFNγ production. As this study and that of Phythian-Adams *et al* are the only to have assessed the impact of DC depletion during established chronic infection, further investigations

will be useful to clarify the role of DCs in a variety of chronic infectious disease settings.

In contrast to the relatively intact effector T cell polarisation, the capacity for $CD4^+$ T cell IL-10 production, either alone or alongside IFNγ, was reduced after ablation of $CD11c^+$ cells, suggesting that T cells with this phenotype were dependent on cDCs for their expansion and/or maintenance, although the mechanisms behind this are not clear. The first possibility is that the cDC cytokine profile is key to their generation, and that the ablation of cells which express IL-10 and IL-27 during infection (cf. Chapter 3) prevents the development of $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells. Although IL-10 has been reported as a key factor for the differentiation of $IL\text{-}10^+$ regulatory Tr1 cells [487, 582, 583], it would appear that $IFN\gamma^+IL\text{-}10^+$ T cells can differentiate independently of IL-10 [162, 528] and so the loss of $IL\text{-}10^+$ cDCs after DTx administration is unlikely to impact upon their expansion.

IL-27 has a well documented role in the generation of $IL\text{-}10^+$ T cells [255, 259, 260, 262, 486], and so the ablation of IL-27-producing cDCs could be expected to prevent the expansion of T cells with this phenotype. However, levels of serum IL-27p28 were unaffected by ablation of $CD11c^+$ cells, suggesting that other cell types are also capable of IL-27 production during infection and that IL-27 would thus be available for the polarisation of T cells toward this phenotype in the absence of cDCs. However the maintenance of systemic IL-27 levels does not rule out the lack of $IL\text{-}27^+$ cDCs as a mechanism underlying the reduced expansion of $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells, as it is possible that local production of IL-27 was affected by CD11c ablation; further work will be required to address this.

The reduction in both serum IL-10 and cDCs expressing IL-10 may have also affected inflammatory cytokine production by other immune cell types, thus impacting on T cell polarisation. For example IL-6 from transferred BMDCs is known to inhibit the generation of $CD4^+IFN\gamma^+IL\text{-}10^+$ T cells during therapeutic intervention of chronic *L. donovani* infection [391] and IL-6 production is directly inhibited by IL-10 [524, 584]. As such, the reduced IL-10 production after $CD11c^+$ cell ablation during infection may have allowed greater expression of IL-6, thereby inhibiting $CD4^+IFN\gamma^+IL\text{-}10^+$ T cell expansion. However, enhanced clearance of *L. donovani* has been reported in *Il6*$^{-/-}$ animals [585], suggesting that increased IL-6 levels may actually be detrimental to the host response.

In addition to reduced IL-10 production by $CD4^+$ T cells, ablation of CD11c-expressing cells also reduced serum levels of IL-10, indicating that both $IL\text{-}10^+$ cDCs and $CD4^+$ T cells, which are both significantly reduced in frequency after DTx administration, contribute to systemic IL-10 levels during infection. High levels of serum IL-10 during infection are well documented in clinical studies, with these levels decreasing significantly after successful drug treatment [586-590]. Serum from patients with active disease has also been shown to directly inhibit killing of *L. donovani* amastigotes by human macrophages *in vitro*- a mechanism strictly dependent upon IL-10 [390]. Furthermore, evidence for the direct suppression of NO production and parasite killing by IL-10 *in vitro* [591, 592] and *in vivo* [593] suggests a dominant role for IL-10 in suppressing NO-mediated parasite clearance during persistent infection. Supporting this evidence, the ablation of $IL\text{-}10^+$ CD11c-expressing cells and the subsequent reduction in $IL\text{-}10^+$ T cell expansion in this study led to a concomitant increase in splenic NO levels and reduced parasite burdens in this organ; despite the slight decrease in the frequency

of splenic $CD4^+IFN\gamma^+$ T cells. As such, it would appear that the outcome of chronic parasitisation in the spleen is determined by levels of IL-10, which when reduced, allows the remaining IFNγ to initiate NO-dependent parasite clearance. As discussed later in this study, multiple $IL\text{-}10^+$ cell types appear to be capable of maintaining parasite persistence and pathology during infection; however further studies utilising strains of mice with cell-specific *Cre*-mediated ablation of the IL-10R will be required to determine whether a particular cell type requires sensitivity to IL-10 for disease establishment and/or progression.

As neutrophils are capable of internalising and killing *L. donovani* [594] and contribute to early control of parasite number [595], can act as functional APCs via expression of MHCII [596, 597] and a B7-like molecule [598] and may modulate T cell cytokine [599] and chemokine [600] production during experimental leishmaniasis, it was important to address the potential issues introduced to the study by the influx of neutrophils observed after DTx administration. Neutrophil depletion was therefore carried out using 1A8, a Ly6G-specific antibody, which selectively depletes neutrophils and leaves other cell populations, such as inflammatory monocytes, intact [601].

Ablation of neutrophils did not significantly alter the capacity for $CD11c^+$ depletion to inhibit the expansion of $IFN\gamma^+IL\text{-}10^+$ T cells, nor did it impact upon T cell polarisation in CD11c-sufficient animals; despite the previously reported roles for neutrophils in modulating T cell cytokine production. Neutrophil influx as a result of DTx administration did not explain the reduced splenic pathology in CD11c-depleted animals, as 1A8 treatment did not prevent the reduced splenomegaly or enhanced NO production observed after CD11c depletion (not shown). Parasite burdens were higher

in 1A8 treated animals when $CD11c^+$ cells were also depleted; however this is probably due to the dramatically enhanced parasite burdens seen after neutrophil depletion in chronically infected C57BL/6 mice (not shown and Yurkudal *et al*, in preparation). Therefore it is unlikely that the dramatic changes in pathology and $CD4^+IFN\gamma^+IL\text{-}10^+$ T cell frequency observed after ablation of $CD11c^+$ cells are caused by an influx of neutrophils as a result of DTx administration.

A major observation during CD11c ablation experiments was the loss of $CD11c^{int}$ cells in addition to $CD11c^{hi}MHCII^{hi}$ cDCs after administration of DTx. On careful examination of many of the published studies deploying similar CD11c depletion strategies, it is apparent that many DTR systems result in the loss of splenic $CD11c^{int}$ cell populations (for example see [506]). This fact has not been addressed previously, and so transfers of $CD11c^{hi}MHCII^{hi}$ cDCs and $CD11c^{int}$ populations isolated from infected donors into infected, CD11c-depleted recipients were performed. Transfer of the $CD11c^{int}$ population was particularly important as $CD45RB^+$ regulatory DCs and splenic NK cells are known to be significant sources of IL-10 during *L. donovani* infection [172, 392], express intermediate levels of CD11c and therefore comprise part of the $CD11c^{int}$ population depleted in this study.

Whilst transfer of DCs back into CD11c-depleted animals has been reported previously, these studies used *in vitro*-derived BMDCs [602, 603] or minimally purified splenic DCs [576] as the transferred population, in most cases generated / isolated from naïve hosts. As such, this study is the first to attempt to reconstitute the $CD11c^{hi}MHCII^{hi}$ compartment with splenic cDCs sorted to high purity from mice with ongoing infection and thus in an as physiologically relevant state as possible. However one potential

caveat is the number of cells available for transfer; transferred cells were ~10 fold fewer than the number of cells present in an unmanipulated, infected animal. Despite this, significant effects on splenic pathology were observed after transfer, although no donor cells were detectable by flow cytometry 7 days later. This is perhaps unsurprising for the transferred splenic cDC population, which have a half-life considerably shorter than 7 days [55, 56], but the $CD11c^{int}$ population will have contained cDC precursors [47], albeit at an extremely low frequency, and so may have been expected to differentiate into cDCs after transfer. Nevertheless the effects of cDC transfer on pathology were considerable, indicating the potency of these cells to manipulate immune responses. Highlighting this fact, transfer of only 30,000 $CD11c^{hi}$ cDCs isolated from draining lymph nodes of mice responding to house dust mite antigen has been reported to modulate several parameters of systemic immune function [604].

Of note, $CD11c^{hi}MHCII^{hi}$ and $CD11c^{int}$ populations from infected mice were equally effective at reconstituting the pathological response in the spleen after transfer, although cDCs were the only population which facilitated the expansion of $IFN\gamma^{+}IL\text{-}10^{+}$ $CD4^{+}$ T cells. As the $CD11c^{int}$ population will have contained NK cells, this is line with previous data showing that NK cells produce IL-10 during infection and when transferred from infected mice, exacerbate disease [392]. Furthermore, although it was not directly addressed in the previous study [172], the reconstitution of disease by $CD11c^{int}$ cells suggests that $CD11c^{lo}CD45RB^{+}$ IL-10-producing DCs may also contribute directly to disease progression. However, the $CD11c^{int}$ population transferred here will have contained both NK cells and $CD11c^{lo}$ DCs, so it is not possible to distinguish between the effects of each cell type.

The reconstitution of the progressive splenomegaly, parasite burden and serum IL-10 levels by $CD11c^{int}$ cells occurred despite maintaining a reduced frequency of $IL\text{-}10^{+}$ $CD4^{+}$ T cells, suggesting that IL-10 from T cells does not greatly impact on parasite burden, splenic pathology or systemic levels of IL-10. This is consistent with previous work showing that dramatic improvements in parasite persistence and splenic pathology can be achieved by therapeutic infusion of LPS-activated BMDCs, irrespective of whether the splenic IL-10-producing $CD4^{+}$ T cell frequency is reduced or maintained [391], in addition to the limited functional role of IL-10 production by $IFN\gamma^{+}$ T cells in preventing Th1 responses after experimental vaccination against *L. major* [528]. Studies involving the adoptive transfer of IFNγ and IL-10 co-producing cells isolated from infected mice would be required to address the functional significance of T cells with this phenotype in relation to disease progression and pathology.

The paradoxical finding that $CD11c^{+}$ cells, including $CD11c^{hi}$ cDCs, negatively regulate the host response to *L. donovani* is intriguing, given that these cells are critical for initiating effector immune responses and usually considered entirely beneficial during the host response to infection. However, a basis for DC dysfunction during chronic infection is well established, as the infusion of BMDCs leads to a dramatic reduction in parasite burden and splenic pathology (Moore *et al,* in preparation and [313, 391, 605, 606]), suggesting that overwhelming the endogenous, functionally impaired DC populations with fully activated DCs is enough to 'kick start' the response to the parasite. The data presented here support this model, as an alternative approach leading to depletion of the impaired DC populations used in this study allows for a similar enhancement in the response to infection.

Taken together, this data reveals $CD11c^{hi}$ cDCs as the critical cell population involved in the expansion of $IFN\gamma^{+}IL\text{-}10^{+}$ $CD4^{+}$ T cells during chronic *Leishmania donovani* infection *in vivo*. Although cDCs in isolation are sufficient to maintain chronic infection, adoptive transfer of other CD11c-expressing cell populations affected by DTx administration suggests that other immune cell types such as NK cells, $CD11c^{lo}CD45RB^{+}$ DCs or as yet unidentified $CD11c^{int}$ cell populations are also capable of mediating some elements of splenic pathology and disease progression.

In conclusion, this chapter reveals that splenic cDC function during chronic infection is modulated by an auto-regulatory cytokine cascade, involving suppression of IL-12p70 production by autocrine IL-10. This impaired IL-12 production is accompanied by production of IL-27, which inhibits IL-12-induced Th1 polarisation and presumably underlies the impaired capacity for these cells to generate Th1 responses *in vitro*. CD11c depletion and subsequent adoptive transfer approaches revealed a critical role for cDCs in the expansion of $CD4^{+}IFN\gamma^{+}IL\text{-}10^{+}$ T cells and the capacity for cDCs to maintain splenic pathology and chronic infection. IRF7 appeared to be redundant in the regulation of chronic infection in the spleen, however in light of the critical role for IRF7 and the type 1 IFN pathway in regulating the development, cytokine profile and PRR signalling pathways of DCs, the next chapter focuses on the role for this transcription factor in the regulation of cDC development and activation *in vitro* and *in vivo*.

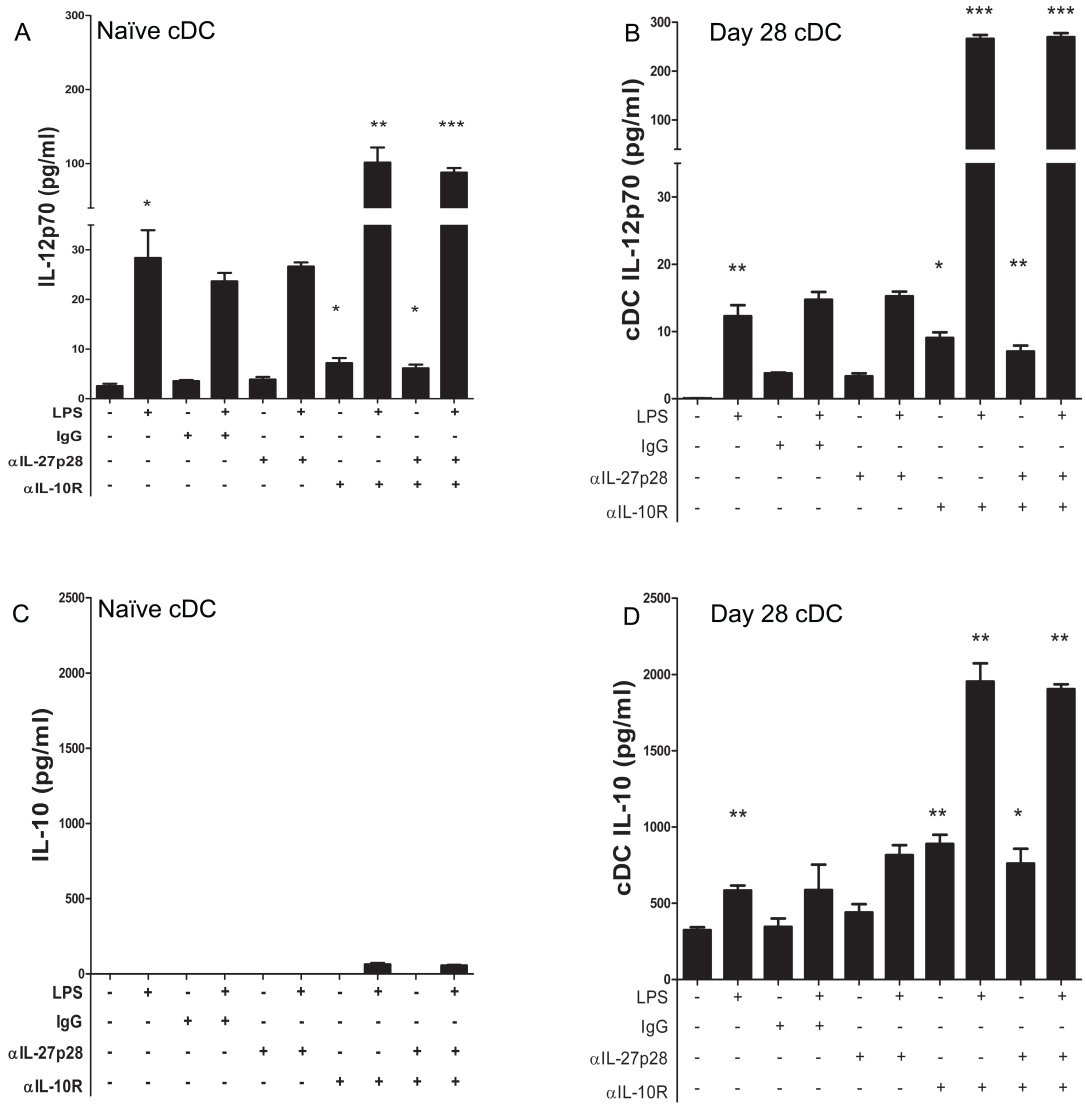

Figure 4.1 Autocrine IL-10 signalling inhibits IL-12p70 production by cDCs. CD11c^{hi} cDCs were sorted from pooled naïve or day 28 infected mice as previously described. cDCs were plated in triplicate at 1x10^6 cells/ml in complete RPMI. Where indicated, LPS (1µg/ml), goat IgG (10µg/ml), αIL-27p28 (10µg/ml) and/or αIL-10R (1.3µg/ml) were added to to give the indicated final concentration. Cells were cultured for 24 hours and resulting supernatants assessed by ELISA for presence of IL-12p70 (**A** & **C**) and IL-10 (**B** & **D**). Data are from one experiment (naïve cDCs) or representative of two experiments (day 28 cDCs) and show the mean concentration of the indicated cytokine ±SEM from triplicate wells containing cDCs sorted from 3-4 pooled naïve or day 28 infected mice. *=p<0.05 **= p<0.01, ***=p<0.001 for indicated treatment vs control conditions.

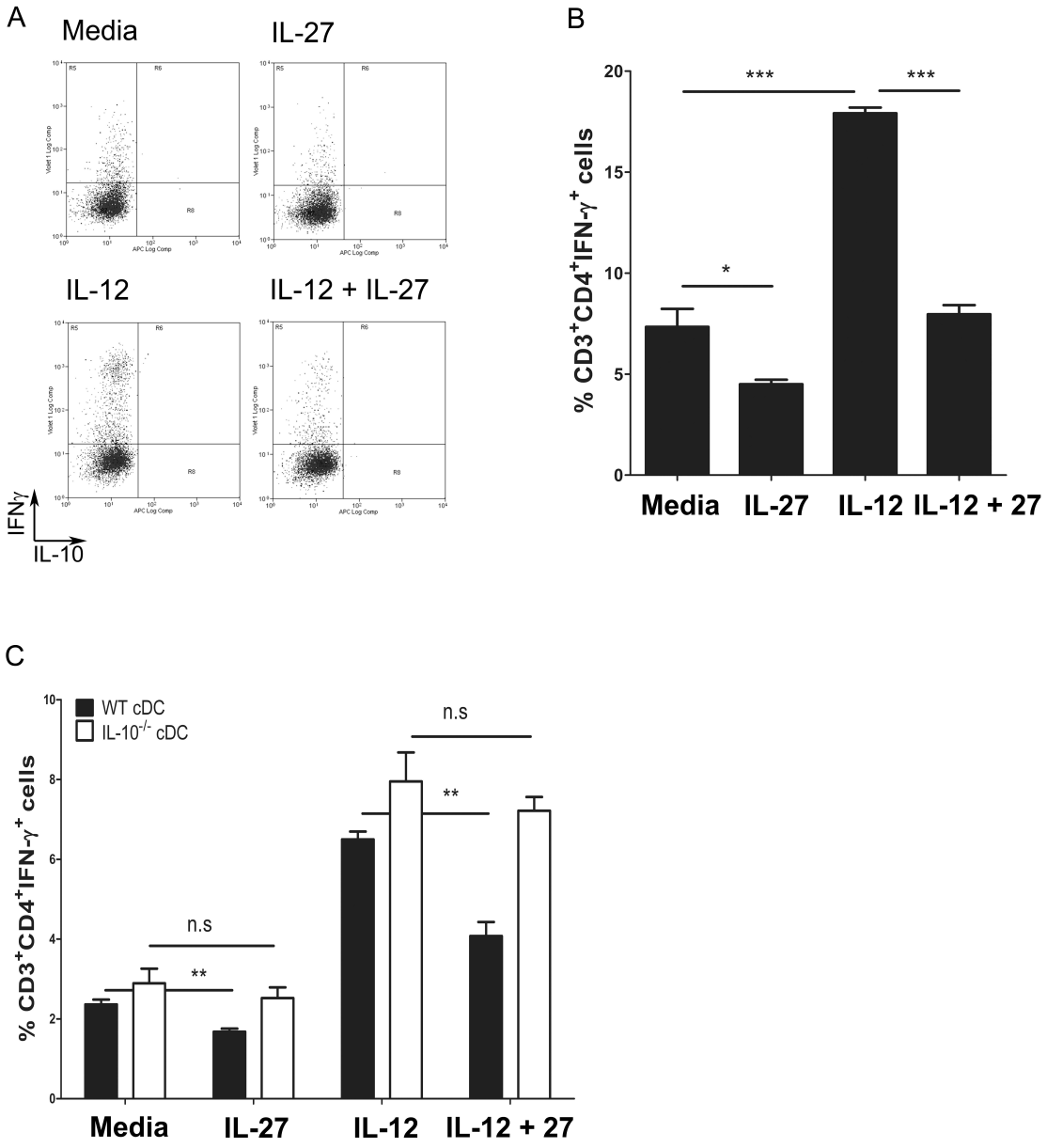

Figure 4.2 IL-27 inhibits IL-12 mediated Th1 induction in vitro by an IL-10-dependent mechanism. $CD11c^{hi}$ cDCs were sorted to high purity from naïve C57BL/6 or naïve B6.*Il10*$^{-/-}$ mice. cDCs were cultured at a 1:5 ratio with sorted $TCR\beta^{+}CD44^{lo}CD62L^{hi}$ OTII.RAG2$^{-/-}$ T cells in the presence of 5nM $OVA_{(323-339)}$. Where indicated IL-27 (20ng/ml) and/or IL-12 (3ng/ml) were added to media to give the indicated final concentration. Cells were cultured for 5 days, with fresh media, containing cytokines as appropriate, being added at day 3. After culture period, cells were restimulated with PMA and Ionomycin, in the presence of Brefeldin A, and assessed by intracellular cytokine staining for $CD3\varepsilon^{+}CD4^{+}$ T cell expression of IFNγ and IL-10. **A** shows representative flow plots of $CD3\varepsilon^{+}CD4^{+}$ gated cells from cultures containing cDCs from C57BL/6 mice. **B** and **C** show mean frequency ± SEM of $IFN\gamma^{+}$ T cells in cultures containing cDCs from C57BL/6 or C57BL/6 vs B6.*il10*$^{-/-}$ mice, respectively. Data are from one experiment. *=p<0.05 **= p<0.01, ***=p<0.001

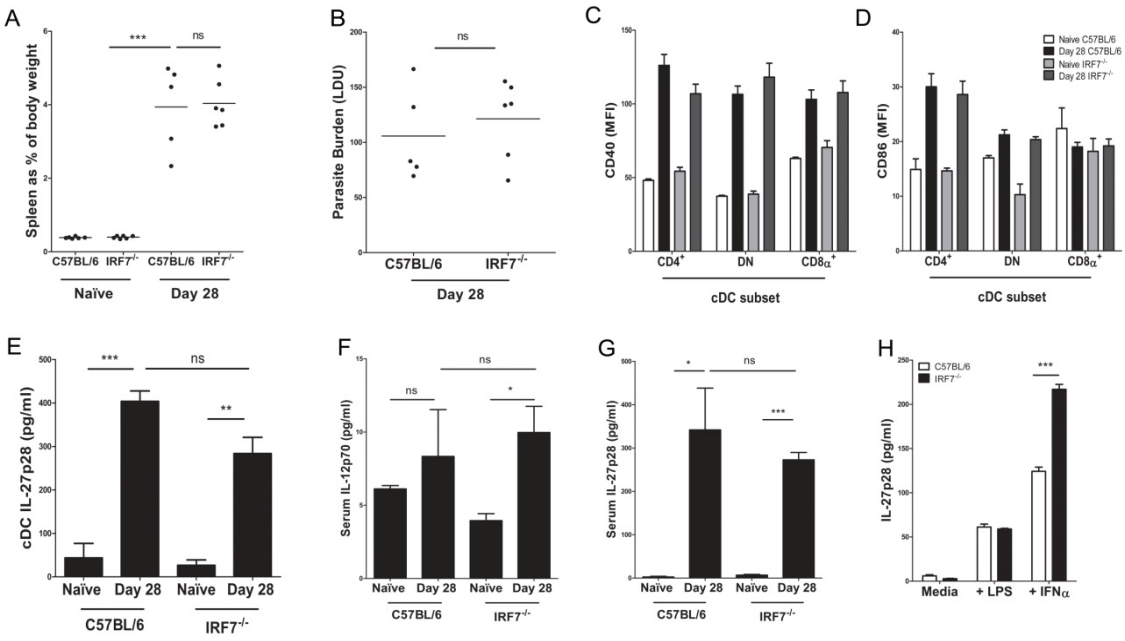

Figure 4.3 IRF7 is not required for IL-27 production *in vitro* or *in vivo* and splenic responses to chronic *Leishmania donovani* infection are unaffected in B6.*Irf7*$^{-/-}$ hosts. C57BL/6 and B6.*Irf7*$^{-/-}$ mice were infected with *L. donovani* and infection allowed to develop until day 28. **A** shows splenomegaly and **B** spleen parasite burden, determined as previously described. **C** and **D** show expression of CD40 and CD80 by cDC subsets from day 28 infected and naïve age-matched control animals, determined by flow cytometry. CD11c^{hi}MHCII^{hi} cDCs were sorted from pooled spleens of naïve and day 28 infected C57BL/6 and B6.*Irf7*$^{-/-}$ mice and cultured at 1x10^{6} cells/ml for 24 hours. **E** shows levels of IL-27p28 protein in culture supernatants, determined by ELISA. **F** and **G** show levels of of IL-27p28 and IL-12p70 in sera from naïve and day 28 infected mice of both strains assessed by ELISA. cDCs were sorted from naïve C57BL/6 and B6.*Irf7*$^{-/-}$ mice and cultured for 24 hours in the presence of 1μg/ml LPS or 1000 U/ml IFNα. **H** shows culture supernatants assessed by ELISA for presence of IL-27p28. Data are pooled from two experiments (**A** & **B**), or representative of two experiments (**C** – **H**) and show mean ±SEM from n=3 mice per group, per experiment. *=p<0.05 **= p<0.01, ***=p<0.001

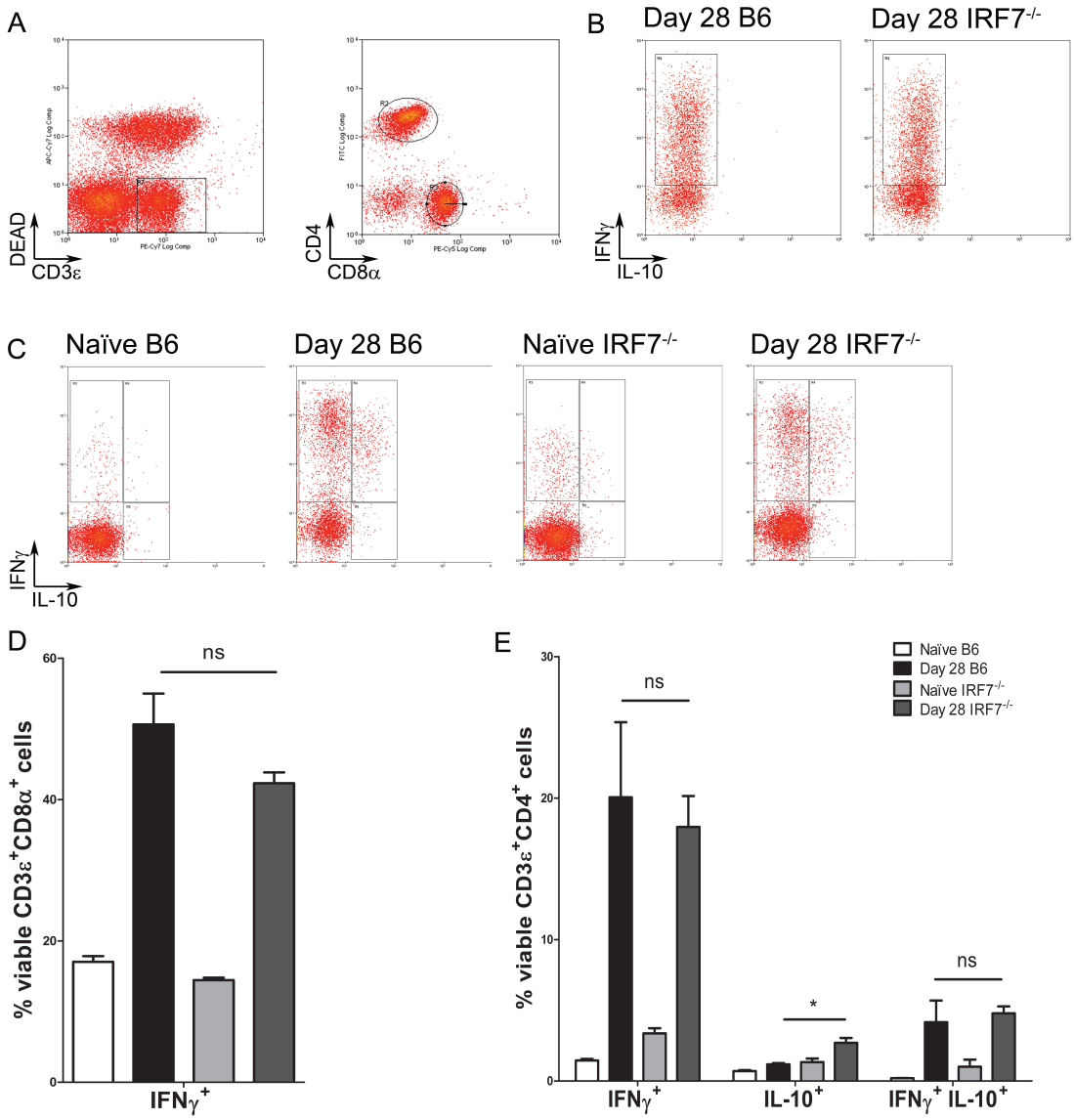

Figure 4.4 Equivalent T cell responses in chronically infected mice deficient in IRF7. Splenocytes from naïve and day 28 infected C57BL/6 and B6.*Irf7*$^{-/-}$ mice were restimulated with PMA, Ionomycin and Brefeldin A as before. **A** shows viable CD3ε^{+} cells, divided into CD4^{+} and CD8α^{+} subpopulations for analysis. Intracellular cytokine staining allowed assessment of IFNγ and IL-10 production by CD8α^{+} (**B** & **D**) and CD4^{+} (**C** & **E**) T cells. **A-C** show representative flow plots, **D** and **E** show mean frequency ±SEM of CD8α^{+} or CD4^{+} T cells with the indicated cytokine producing phenotype. Data are from n=3 mice per group and representative of two separate experiments. *=p<0.05

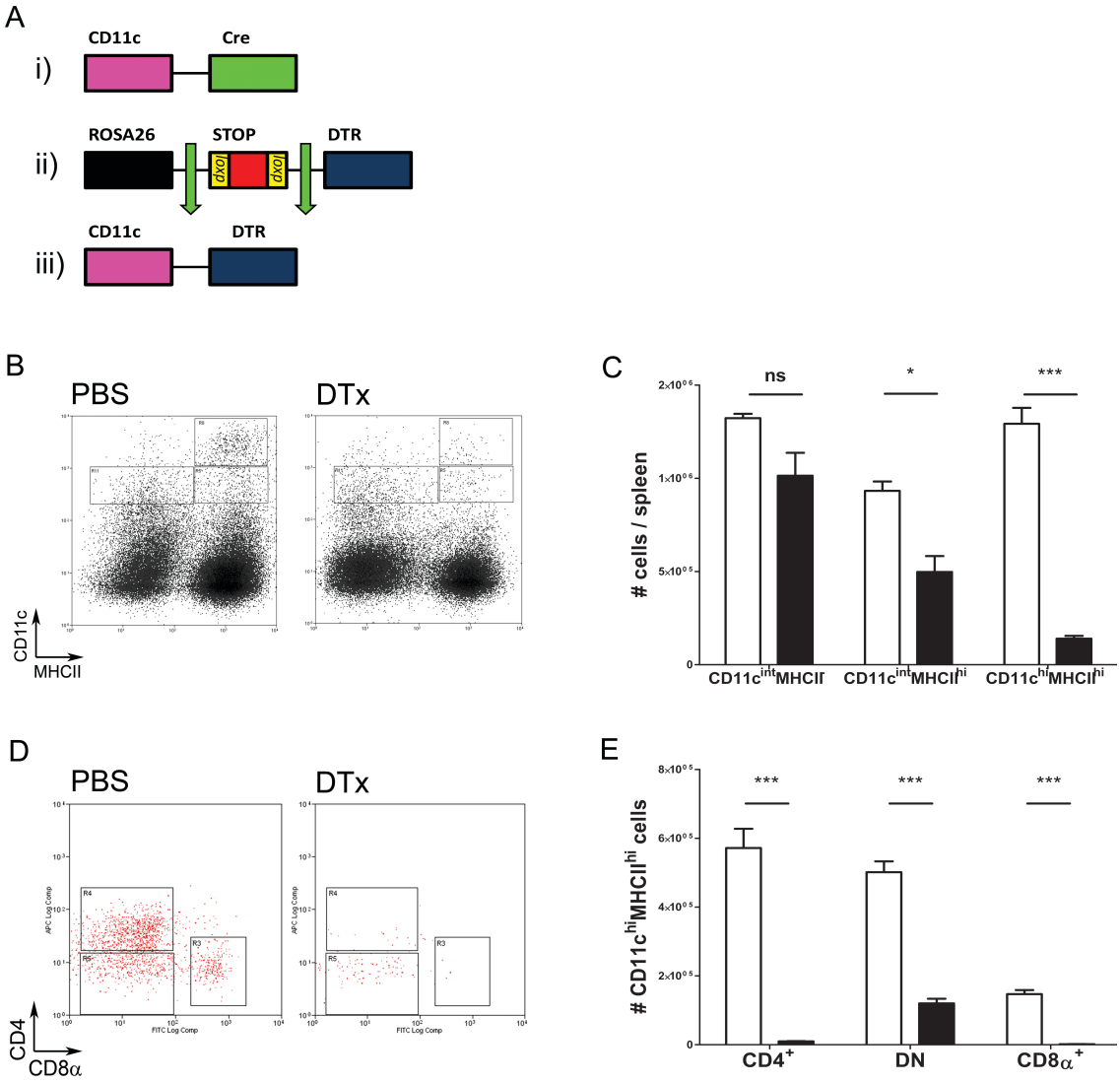

Figure 4.5 Efficient conditional ablation of cDC subsets in CD11c.iDTR mice. CD11c.iDTR mice were generated as described (**Chapter 2.1**). In these mice, expression of Cre recombinase under the control of the CD11c-promoter (**A i**) allows cleavage of *loxP* flanking regions around a STOP sequence inserted into the ROSA26 locus (**A ii**), leading to Diptheria Toxin Receptor (DTR) expression only in those cells with CD11c promoter activity (**A iii**). **B** shows representative flow plots indicating ablation of CD11c-expressing cells in naïve mice after PBS or DTx injection, with numbers of cells in PBS treated (open bars) or DTx treated (closed bars) mice shown in **C**. **D** and **E** show effects of DTx treatment on the number of conventional $CD4^+$, DN and $CD8\alpha^+$ DC subsets (open bars= PBS treated, closed =DTx treated). Dot plots are representative and data in **C** and **E** show mean numbers of indicated cells ±SEM from n=4-5 mice per group and are representative of three individual experiments. *=$p<0.05$, ***=$p<0.001$ for DTx vs PBS treated mice

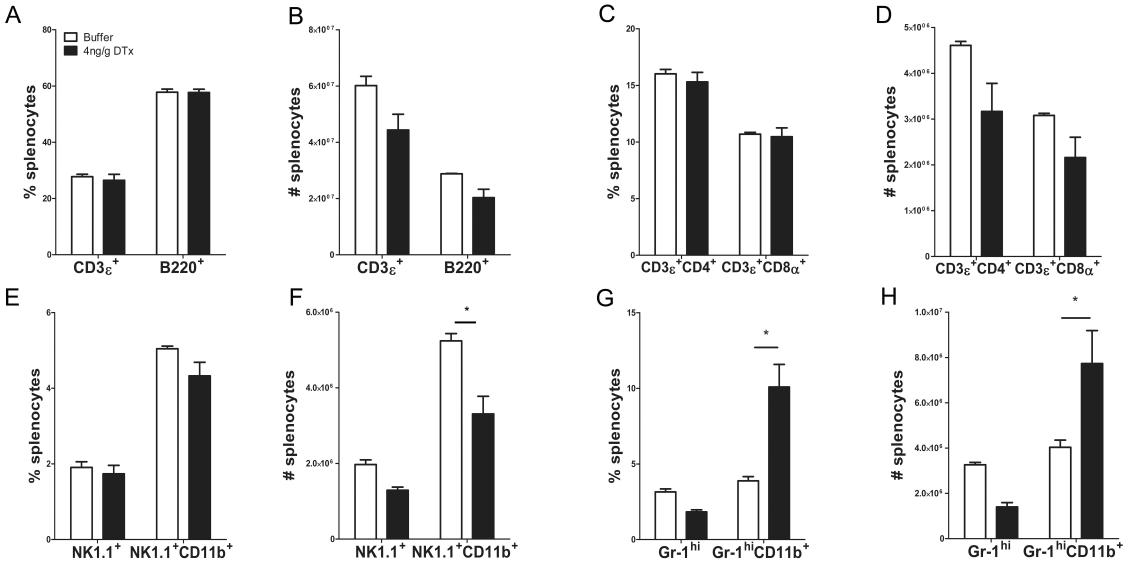

Figure 4.6 Effects of DTx administration on spleen cell composition in CD11c.iDTR mice. Alterations in the frequency and number of splenic T and B cells (**A** & **B**), $CD4^+$ and $CD8\alpha^+$ T cells (**C** & **D**), NK cells (**E** & **F**) and neutrophils (**G** & **H**) were assessed by flow cytometry after administration of PBS (open bars) or DTx (closed bars) to naïve CD11c.iDTR mice at 48hr intervals over a 7 day period. Data are mean ± SEM from 4-5 mice per group and representative of two or three separate experiments. *=$p<0.05$ for DTx versus PBS treated mice.

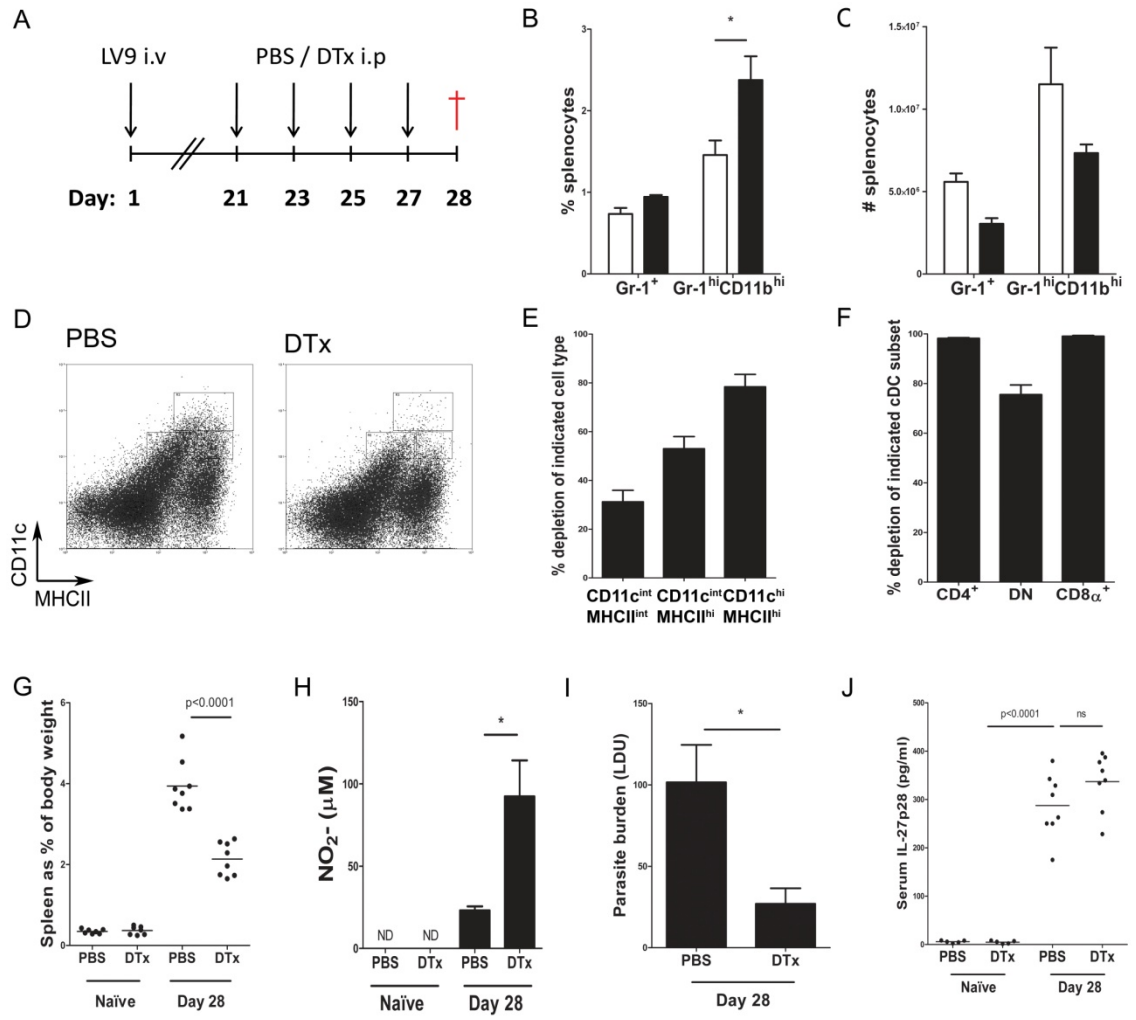

Figure 4.7 Conditional ablation of CD11c$^+$ cells during chronic infection reduces splenic pathology, enhances nitric oxide production and initiates parasite clearance. **A.** Naïve or infected CD11c.iDTR mice received PBS or DTx at 48hr intervals from day 21 until day 28 of infection. **B&C.** Alterations in the frequency and number of neutrophils in PBS treated (open bars) and DTx-treated mice. **D.** Depletion of CD11c-expressing cell populations was determined by flow cytometry. **E** and **F** show depletion levels expressed as a percentage loss of indicated CD11c$^+$ cell populations or cDC subsets. **G** shows the effects of DTx administration on splenomegaly, determined as previously. **H** shows levels of nitric oxide in supernatant of adherent spleen cell cultures after 24 hours in complete RPMI, determined by Greiss assay. **I** shows spleen parasite burdens in PBS or DTx treated infected mice, determined as before. **J** shows levels of IL-27p28 in serum, determined by ELISA. Flow plots in **D** are representative, data in **B**, **C**, **E, F, H** and **I** show mean ±SEM of indicated parameters from n=4 (naïve) or 5 (infected) mice per group and are representative of two experiments. **G** and **J** show mean values from pooled mice from two experiments, n=7/8 per group. *=p<0.05 for DTx vs PBS treated mice.

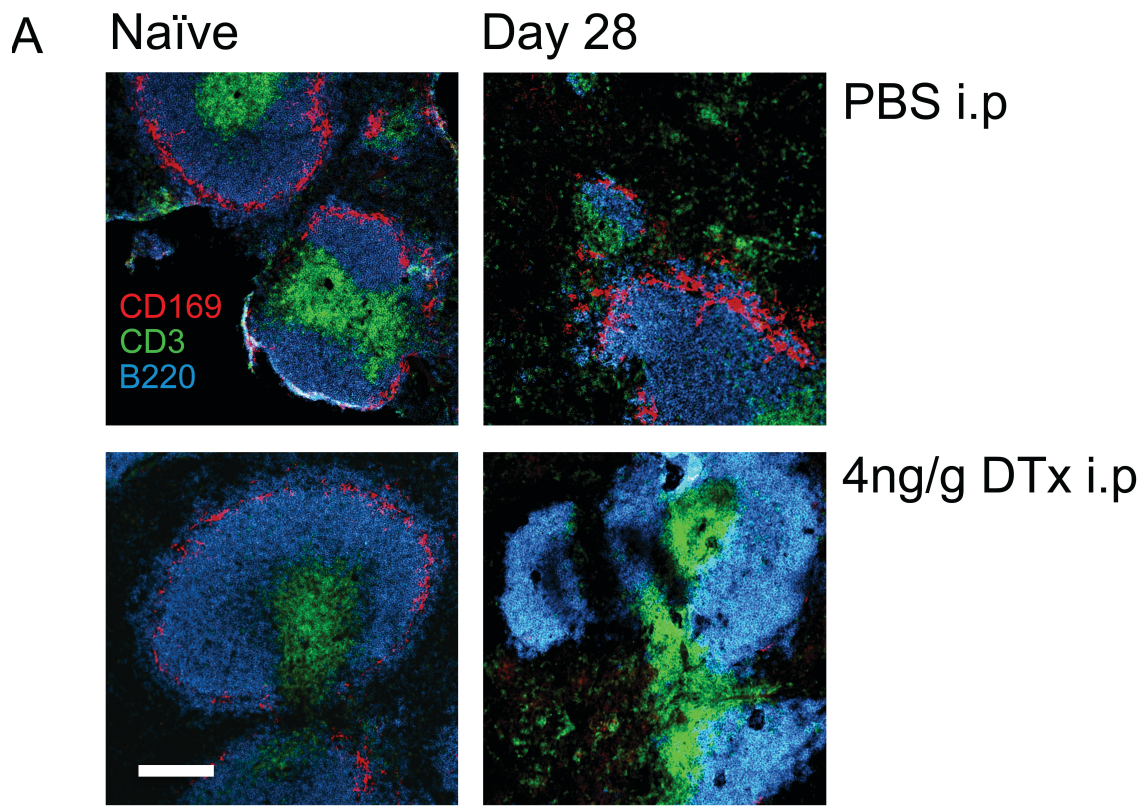

Figure 4.8 Effects of DTx administration on splenic architecture in naïve and chronically infected CD11c.iDTR mice. A. Naïve and infected mice were treated with PBS or DTx according to the schedule previously described. Cryosections were taken from $5mm^3$ blocks of splenic tissue. Sections were assessed for expression of CD169, CD3ε and B220 by confocal microscopy. Representative tissue sections shown. Scale bar = 100μm.

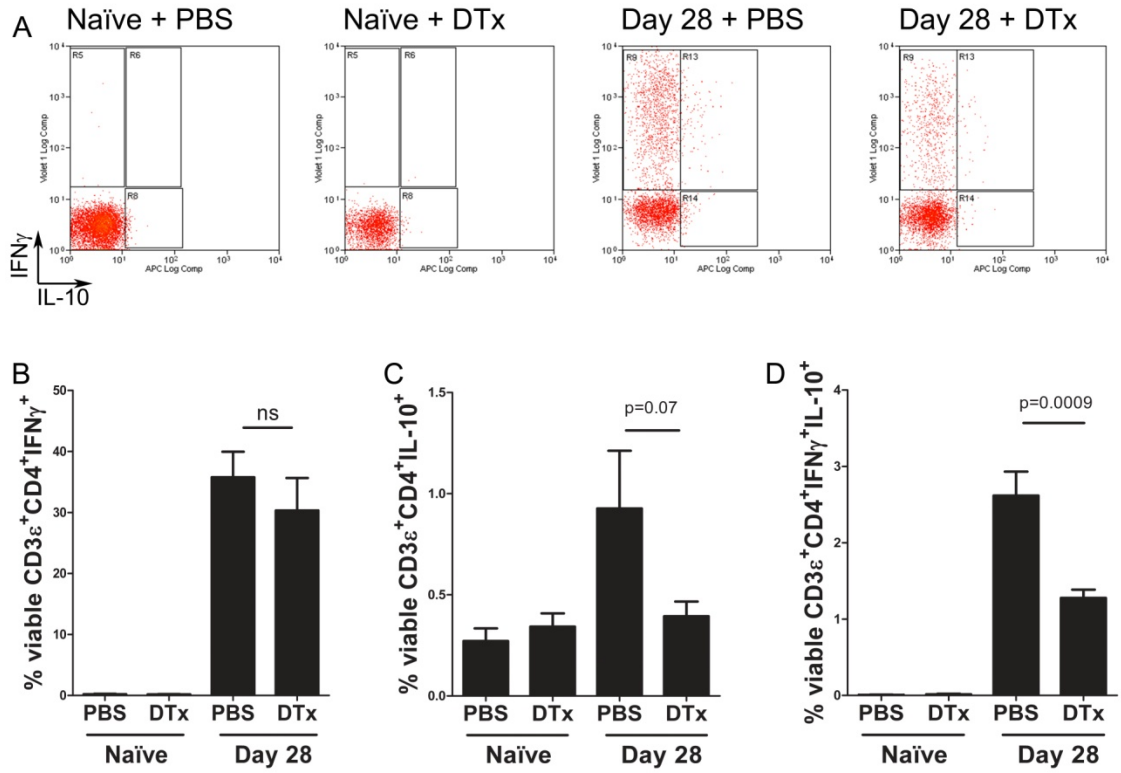

Figure 4.9 Ablation of CD11c$^+$ cells during chronic infection impairs the generation of IL-10$^+$ and IFNγ$^+$IL-10$^+$ antigen-specific T cells *in vivo*. Naïve or infected CD11c.iDTR mice received PBS or DTx at 48hr intervals from day 21 until day 28 of infection, as described previously. Splenocytes were isolated and restimulated with *L. donovani* antigen-pulsed BMDCs, in the presence of Brefeldin A. **A** shows cytokine production by CD3ε$^+$CD4$^+$ T cells assessed by flow cytometry after intracellular cytokine staining. The frequencies of antigen-specific IFNγ$^+$ (**B**), IL-10$^+$ (**C**) and IFNγ$^+$IL-10$^+$ (**D**), cells were quantified. Representative flow plots are shown in **A** , data in **B**, **C** and **D** show the mean frequency of T cells with indicated phenotype ±SEM in mice from two pooled experiments, n=7/8 per group.

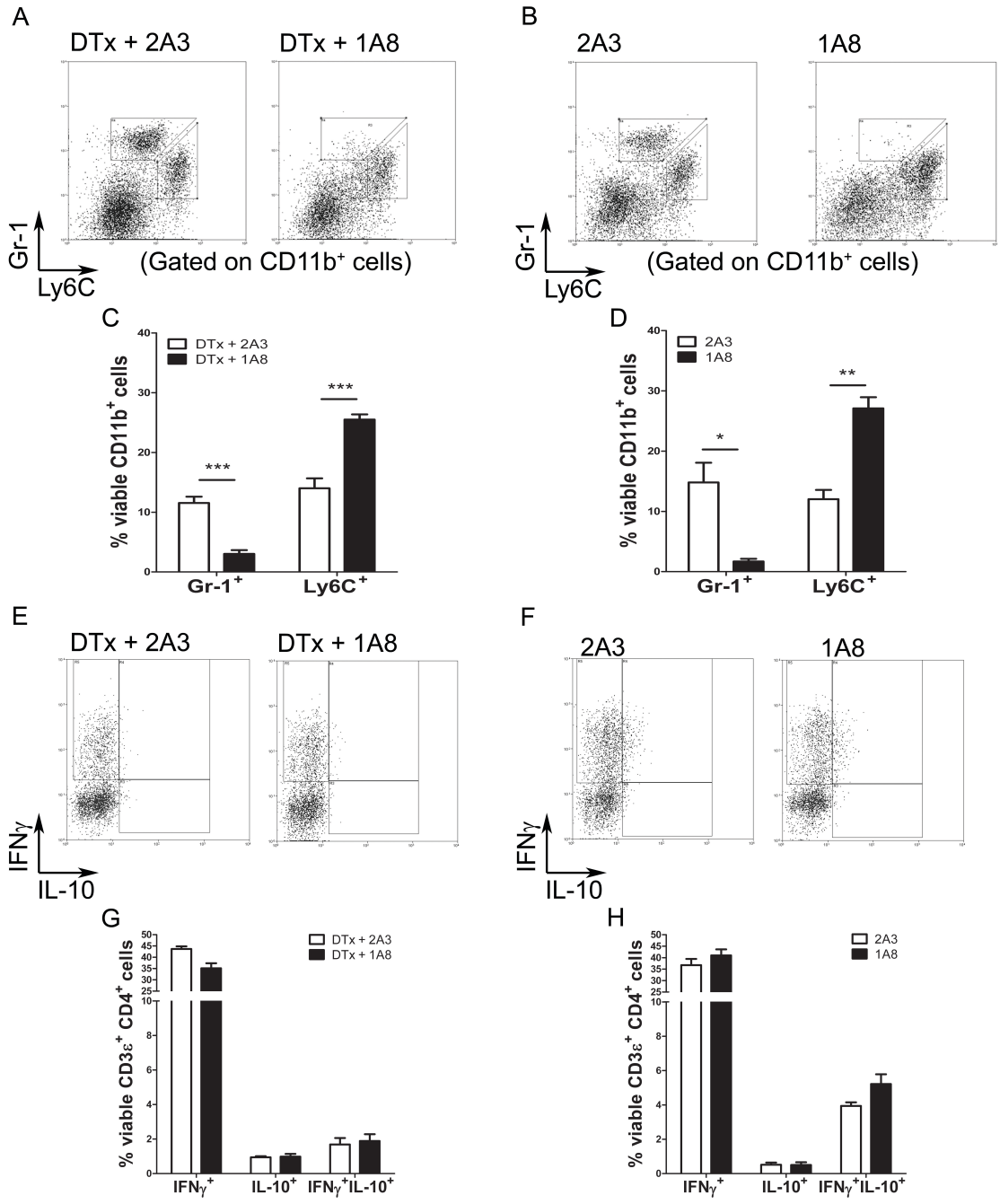

Figure 4.10 Neutrophil influx due to DTx administration does not account for altered CD4$^+$ T cell phenotype after ablation of CD11c$^+$ cells during chronic infection. Infected C57BL/6 or CD11c.iDTR (undergoing CD11c-depletion to the schedule as previously described) mice were treated i.p at days 20, 23 and 26 with 250µg Ly6G-specific 1A8 or isotype control 2A3 antibodies. **A** and **B** show representative flow plots depicting specific depletion of CD11b$^+$Gr-1$^+$ neutrophils (plots previously gated on CD11b$^+$ cells). **C** and **D** show alterations in the frequency of CD11b$^+$Gr-1$^+$ and Ly6C$^+$ cells after indicated treatment from CD11.iDTR and C57BL/6 mice, respectively. **E** and **F** show cytokine production by CD3ε$^+$CD4$^+$ T cells after restimulation with PMA and Ionomycin. **G** and **H** show quantified T cell cytokine responses after indicated treatments. Flow plots are representative, charts show mean ±SEM. Data are from one experiment with 4 or 5 mice per group.

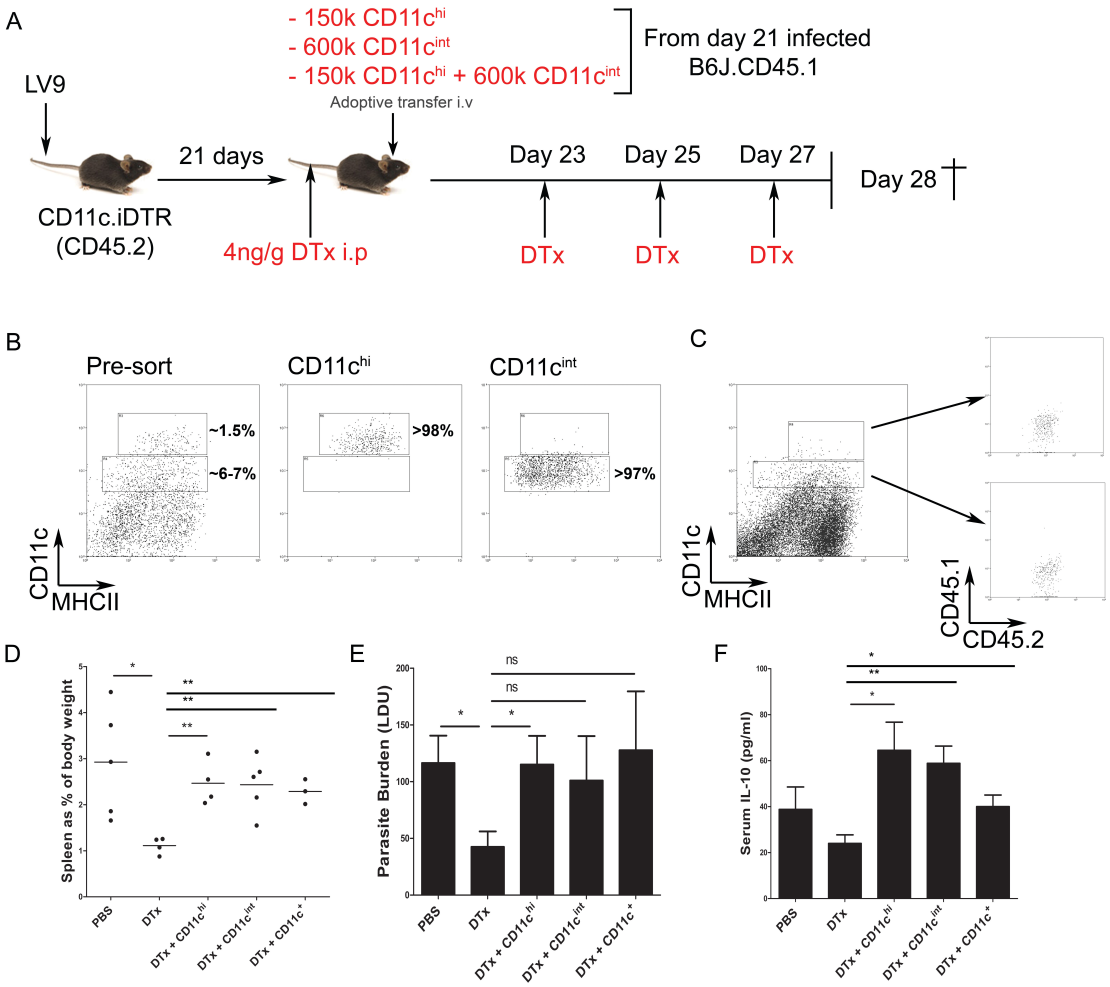

Figure 4.11 Adoptive transfer of CD11c^{+} cells from chronically-infected mice confirms their key contribution to the maintenance of chronic disease. A shows experimental schedule involving 4ng/g DTx administration i.p. at the indicated time points, with adoptive transfer of sorted CD11c-expressing cell populations from congenic, day 21 infected donors at day 21. **B** shows sort gates and post sort purity of CD11c^{hi}MHCII^{hi} and CD11c^{int} populations used for transfer. **C** shows remaining CD11c-expressing cells at day 28, stained for endogenous (CD45.2) and transferred (CD45.1) surface markers. **D** and **E** show splenomegaly and parasite burdens, determined as previously described. **F** shows IL-10 levels in sera from mice at day 28, determined by ELISA. Flow plots are representative, charts show mean ±SEM. Data are from of one experiment with 3-5 mice per group. *=p<0.05, **=p<0.01.

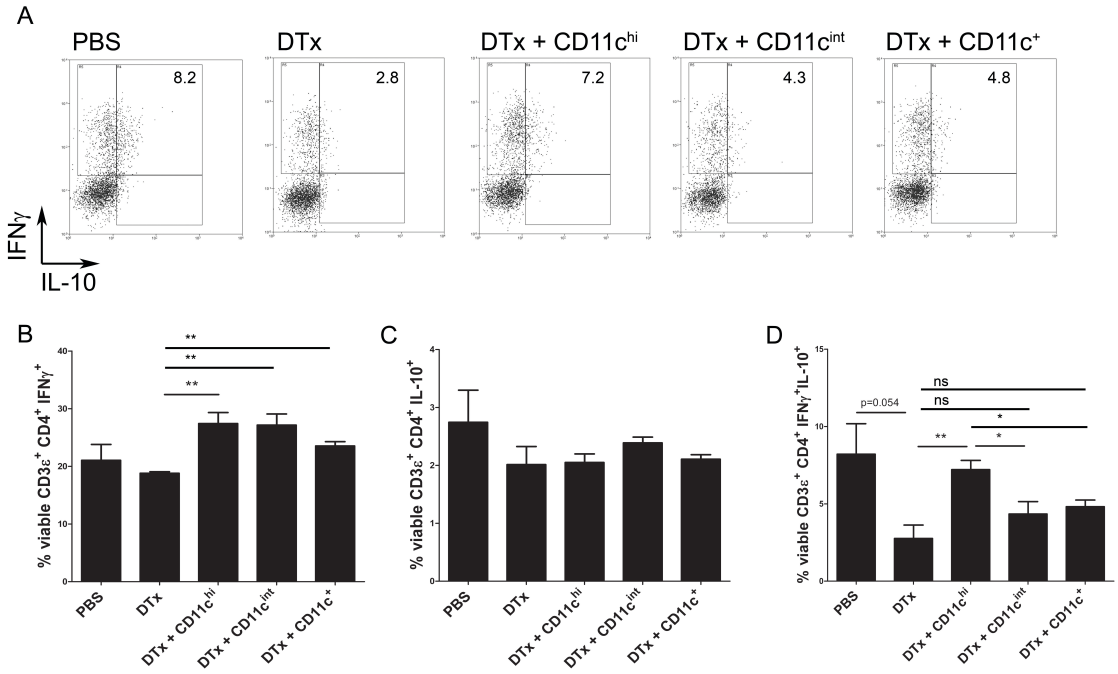

Figure 4.12 CD11c^{hi} cDCs are the major cell type facilitating expansion of splenic CD4$^+$IFNγ$^+$IL-10$^+$ T cells during chronic infection *in vivo*. **A** shows representative flow plots indicating cytokine production by CD3ε$^+$CD4$^+$ T cells from mice as in **4.11** after restimulation with PMA and Ionomycin. **B**, **C**, and **D** show the mean percentages ±SEM of IFNγ$^+$, IL-10$^+$ and IFNγ$^+$IL-10$^+$ CD3ε$^+$CD4$^+$ T cells in the indicated groups. Data are from one experiment with 3-5 mice per group. *=p<0.05, **=p<0.01

Chapter 5: IRF7 regulates the TLR2-induced activation of splenic cDCs *in vivo*.

5.1 Introduction

Interferon regulatory factor (IRF) 7, a member of the IRF family of transcription factors, is the 'master regulator' of type I interferon (IFN)-dependent immune responses and underpins their critical role in host defence [547]. However, IRFs are also essential for the full development of many components of the immune system [607], including dendritic cells. Splenic CD8α^+ cDC development is exquisitely dependent on the expression of IRF8, with *Irf8*$^{-/-}$ mice entirely lacking this subset [608, 609]; a developmental deficiency that can be rescued by retroviral transduction of bone marrow precursors with IRF8 during DC development *in vivo* [610]. Differentiation of splenic CD4$^+$ cDCs depends on IRF4 [611, 612], whereas DN cDCs express and at least partially rely on both IRF4 and IRF8 for their full development [611]. Non-lymphoid DCs such as Langerhans cells and dermal DCs also require IRF8 expression for normal development *in vivo* [613]. In addition, *Irf2*$^{-/-}$ mice selectively lack splenic CD4$^+$ cDCs and epidermal DC subsets [614], whereas IRF1-deficient animals have a reduced number of splenic CD8α^+ cDCs [615]. No study has yet established whether IRF7 is required for full splenic cDC development *in vivo*.

In addition to playing a fundamental role in DC development, type I IFN and IRFs modulate several key elements of APC functionality. IRF2 is required for efficient IL-12 production [616] and IRF1 and IRF8 directly enhance IL-12 production by interaction with promoters of the p40 [617, 618] and p35 [619] subunits, respectively.

Furthermore, IL-12p70 expression by human monocyte-derived DCs appears to be dependent on autocrine IFNα/β signalling, involving upregulation of IRF7 and IRF8 [620], indicating that synergy between several IRF family members is required for full IL-12 production in both mouse and man. IRF1 appears to have additional roles in regulating DC cytokine production, with DCs generated from *Irf1*$^{-/-}$ mice showing impaired TNFα and IL-12 expression and instead producing high levels of IL-10 and TGFβ, with a corresponding loss of T cell polarising capacity *in vitro* [615].

Type I IFN are known to augment costimulatory molecule expression by DCs and monocytes in mice and humans [621-624], but the specific IRFs involved are less clearly defined. PD-L1 and CD40 expression on endothelial cells is critically dependent on IRF1 [625, 626], with this transcription factor also required for in CD80 expression by monocytes *in vitro* - a situation where IRF7 appears to be redundant [627]. Nevertheless, there is some evidence for an interaction between IRF7 and costimulatory molecules, with an IRF7 binding site identified in the CD80 promoter that regulates expression in response to LPS stimulation of human monocytes [628]. Furthermore, large scale analysis of genes regulated by IRF7 in response to viral infection have identified CD80 as a potential target of this transcription factor in an *in vitro* system, suggesting a link between IRF7 and expression of certain costimulatory molecules [629]. However, the potential for IRF7 to regulate costimulatory molecule expression by splenic cDCs is currently unclear.

Several IRFs have a significant impact on the regulation of signalling events after encounter with microbial stimuli and TLR ligation. IRF5, capable of a direct interaction with the key TLR signalling adaptors MyD88 and TRAF6, is essential for cDC and

macrophage expression of pro-inflammatory cytokines after stimulation with a range of TLR ligands [630]. Also capable of interacting with MyD88, IRF4 competes with IRF5 for binding to this adaptor and is thus capable of inhibiting IRF5 activation and preventing subsequent pro-inflammatory cytokine expression induced after TLR ligation [631]. In contrast, IRF8 positively regulates TNFα and IL-6 gene expression by cDCs in response to TLR stimulation, a process initiated by direct interaction with TRAF6 [632, 633]. IRF7 similarly interacts with MyD88 and TRAF6, with these interactions critical for its activation and the subsequent production of IFNα after TLR stimulation [634-636]. In addition, a direct dependence on IRF1 and IRF7 for IFNβ production as a result of TLR2 ligation has also been reported [637].

In light of these previous observations, this chapter aims to investigate whether IRF7 is required for normal splenic cDC subset development *in vivo*, in addition to extending our knowledge of the complex interactions between IRFs and TLRs by characterising the role for IRF7 in the regulation of splenic cDC activation in response to microbial stimuli *in vitro* and *in vivo*.

5.2 Results

5.2.1 IRF7 deficiency does not affect spleen cell composition

Mice deficient in a range of IRFs have profound defects in the development of several immune cell compartments (reviewed in [607]). To determine any impact of IRF7 deficiency on spleen cell development, cells from collagenase-digested splenic tissue of C57BL/6 and B6.*Irf7*$^{-/-}$ mice were assessed by flow cytometry for expression of a range of cell-specific surface markers. The frequency of CD3ε^{+} T cells was equivalent in wildtype and IRF7-deficient animals, at 11.26 ±0.47% and 10.15 ±0.14%, respectively (**5.1A**). A lack of IRF7 did not affect the composition of the CD3ε^{+} compartment, with CD3ε^{+}CD4^{+} cells comprising 52.03 ±1.04% of T cells in C57BL/6 mice and 50.15 ±0.76% in IRF7-deficient animals (**5.1B**). CD8α^{+} T cells were similarly unaffected by the absence of IRF7, making up 34.79 ±0.76% and 35.09 ±0.72% of splenic CD3ε^{+} cells in wildtype and B6.*Irf7*$^{-/-}$ mice, respectively. The frequency of CD19^{+}MHCII^{+} splenic B cells was unaffected by a lack of IRF7, with this population comprising 47.77 ±2.42% of C57BL/6 splenocytes and 52.12 ±3.40% in spleens of B6.*Irf7*$^{-/-}$ animals (**5.1C**). CD11b^{+}Gr-1^{int} monocytic cells were at an equivalent frequency in wildtype and IRF7-deficient mice, at 1.79 ±0.17% and 2.02 ±0.07% of splenocytes, with CD11b^{hi}Gr-1^{hi} neutrophils slightly increased in the absence of IRF7, at 1.02 ±0.21% and 1.54 ±0.37% in spleens of C57BL/6 and B6.*Irf7*$^{-/-}$ mice. Therefore, a lack of IRF7 does not substantially alter the composition of splenic immune cells in the steady state

5.2.2 Faithful splenic cDC development and equivalent steady-state TLR2 expression in the absence of IRF7

As several IRF family members are critical for full cDC subset development [608, 609, 611-615], it was important to determine whether the splenic $CD11c^{hi}MHCII^{hi}$ compartment was intact in steady-state IRF7-deficient mice. Flow cytometric analysis of collagenase-digested spleen tissue from C57BL/6 and B6.*Irf7*$^{-/-}$ mice revealed comparable populations of $CD11c^{hi}MHCII^{hi}$ cells (**5.2*A***). These cells comprised 1.15 ±0.06% of splenocytes in C57BL/6 mice and 0.96 ±0.08% in B6.*Irf7*$^{-/-}$ mice; equivalent to $1.05x10^6$ ±$1.72x10^4$ and $8.96x10^5$ ±$1.02x10^5$ cells, respectively (**5.2B**). Assessing the $CD11c^{hi}MHCII^{hi}$ compartment for expression of CD4 and CD8α allowed the frequencies of cDC subsets to be determined in wildtype and IRF7-deficient animals (**5.2C**). $CD4^+$ cDCs were the most abundant subset in both strains, at 42.32 ±1.01% and 40.21 ±2.43% of $CD11c^{hi}MHCII^{hi}$ cells in C57BL/6 and B6.*Irf7*$^{-/-}$ mice (**5.2D**). The DN subset made up 18.57 ±0.62% of wildtype cDCs, and 18.12 ±0.81% of these cells in B6.*Irf7*$^{-/-}$ mice, whereas the $CD8\alpha^+$ cDC subset comprised 17.20 ±0.38% and 16.49 ±0.79% of $CD11c^{hi}MHCII^{hi}$ cells in steady-state wildtype and IRF7-deficient mice, respectively.

To determine whether IRF7 deficiency altered the capacity for TLR expression by cDCs, $CD11c^{hi}MHCII^{hi}$ cDCs from steady-state wildtype and B6.*Irf7*$^{-/-}$ mice were assessed for surface expression of TLR2 (**5.2E**). cDCs from both strains expressed equivalent amounts of TLR2, at 11.27 ±0.64 units on wildtype cDCs and 10.90 ±0.56 on IRF7-deficient cells (**5.2F**). Taken together, this data indicates that IRF7 is not required for cDC subset development *in vivo* and that TLR2 expression by cDCs is not affected by its absence.

5.2.3 IRF7-deficient splenic cDCs are hyperactivated in response to a TLR2 agonist *in vitro*

To begin to assess the contribution of IRF7 to the regulation of TLR signalling in DCs as physiologically relevant as possible, $CD11c^{hi}$ cDCs were sorted to high purity from spleens of C57BL/6 and B6.*Irf7*$^{-/-}$ mice (**5.3A**). Cells were cultured for a total of 24 hours *in vitro* in the presence of the TLR2 agonist PAM_3CSK_4, and monitored at intervals by flow cytometry for levels of activation, as measured by changes in surface expression of MHCII, CD80 and CD86 (**5.3B**). Stimulation of cDCs from both strains led to their activation, as indicated by progressively increasing expression of all three surface markers. However in all cases, cDCs which lacked expression of IRF7 were hyperactivated in response to TLR2 stimulation, with significantly greater fold increases in expression of CD80 (**5.3C**), CD86 (**5.3D**) and MHCII (**5.3E**) when compared with IRF7-sufficient cDCs stimulated in the same way.

This was reflected by significantly greater fold increases in surface expression of CD80 on B6.*Irf7*$^{-/-}$ cDCs at 2, 4, 8, 16 and 24 hours post-stimulation (p.s.) with PAM_3CSK_4, when compared to levels on unstimulated cDCs from the same strain. At 2hr p.s, wildtype cDCs had not upregulated expression of CD80, whereas IRF7-deficient cells had increased expression by 1.28 ±0.04 fold ($p<0.01$). By 4hr p.s, wildtype cDCs had 1.08 ±0.02 fold higher expression of CD80, whereas IRF7-deficient cells had increased expression by 2.10 ±0.02 fold ($p<0.001$). Increased expression of CD80 reached 1.93 ± 0.01 fold on B6.*Irf7*$^{-/-}$ cDCs by 8hrs, before stabilising at increases of 3.57 ±0.11 and 3.79 ±0.12 fold at 16 and 24 hrs p.s; significantly lower than the progressive increases in CD80 expression observed on wildtype cDCs of 3.54 ±0.03 ($p<0.001$), 7.11 ±0.27 ($p<0.01$) and 8.38 ±0.18 ($p<0.01$) fold at these time points.

Enhanced fold changes in surface CD86 expression in response to PAM_3CSK_4 stimulation were also observed on IRF7-deficient cDCs, however these were only significant at 16 and 24 hours post-stimulation. This was reflected by a 21.39 ±0.85 fold increase in surface CD86 on wildtype cDCs at 16h p.s, compared with a 26.49 ±0.77 fold increase ($p<0.05$) on cDCs lacking IRF7 at this time point. Enhanced CD86 expression in the absence of IRF7 was also seen at 24hr p.s, with a 35.14 ±0.53 fold increase in surface CD86 on B6.*Irf7*$^{-/-}$ cDCs compared with a 22.39 ±0.87 ($p<0.01$) fold increase in expression on wildtype cDCs.

MHCII upregulation in response to TLR2 stimulation occurred in a pattern similar to that seen with CD80, with IRF7 deficient cDCs being significantly more activated at all time points post-stimulation. Wildtype cDCs showed fairly modest fold increases in surface expression of 1.53 ±0.10, 1.73 ±0.04, 2.51 ±0.03, 2.52 ±0.08 and 2.32 ±0.01 at 2, 4, 8, 16 and 24 hours post-stimulation. In contrast, B6.*Irf7*$^{-/-}$ cDCs showed significantly higher fold increases in MHCII expression of 2.42 ±0.05 ($p<0.05$), 3.11 ±0.05 ($p<0.01$), 4.35 ±0.01 ($p<0.001$), 5.59 ±0.23 ($p<0.01$) and 7.90 ±0.05 ($p<0.001$) at the same points post-stimulation. Therefore in the absence of IRF7, splenic cDCs become hyperactivated in response to TLR2 stimulation and show significantly exaggerated expression of the costimulatory molecules CD80 and CD86, as well as MHCII.

5.2.4 IRF7 deficiency has a limited impact on splenic cDC responses to TLR3, TLR4 and TLR9 signalling

To determine whether a lack of IRF7 led to enhanced cDC activation in response to diverse TLR stimuli, an identical approach to that outlined previously was undertaken replacing PAM_3CSK_4 with ligands of TLR3 (Poly (I:C), TLR4 (LPS) and TLR9 (ODN1668) and monitoring activation by changes in surface expression of CD80, CD86 and MHCII by flow cytometry at various intervals during *in vitro* culture (**5.4**).

Unlike with TLR2 stimulation, a lack of IRF7 did not substantially alter the expression of costimulatory molecules in response to agonists of TLR3, TLR4 or TLR9. There were no significant differences in the fold increase in expression of CD80 (**5.4A**) or CD86 (5.4*B*) in response to Poly (I:C), with slightly higher fold increases in MHCII expression being observed, although only significant at 24hr p.s. This was represented by a 2.18 ±0.32 fold increase in surface MHCII on wildtype cDCs at 24hr p.s, compared with a 3.42 ±0.16 ($p<0.05$) fold change on B6.*Irf7*$^{-/-}$ cDCs (**5.4C**). Similarly, a lack of IRF7 did not result in exaggerated costimulatory molecule expression in response to TLR4 stimulation, with, at several time points, slightly lower fold increases in expression of CD80 (**5.4D**) and CD86 (**5.4E**) on B6.*Irf7*$^{-/-}$ cDCs, compared to wildtype cells. However, none of these differences were significant. Fold increases in surface MHCII in response to LPS on wildtype and B6.*Irf7*$^{-/-}$ cDCs were equivalent at all time points post-stimulation (**5.4F**).

In response to stimulation of TLR9 with ODN 1668, CD80 expression appeared to be affected by an absence of IRF7, with higher fold increases in CD80 expression on

B6.*Irf7*$^{-/-}$ cDCs at all time points post-stimulation, although only significantly so at 8hr and 16hr p.s. (**5.4G**). Wildtype cDCs showed 2.55 ±1.11 and 2.83 ±0.10 fold increases in surface CD80 at these time points, compared to 6.00 ±0.98 ($p<0.05$) and 8.39 ±1.04 ($p<0.01$) fold increases on cDCs lacking IRF7. In contrast, there was no significant difference in levels of CD86 (**5.4H**) and MHCII (**5.4I**) expression as a result of IRF7-deficiency at any time point after TLR9 stimulation of cDCs. In summary, the data obtained with agonists of TLR3, 4 and 9 showed only a variable and less pronounced capacity for increased activation of IRF7-deficient cDCs, suggesting that IRF7 may be playing a role in regulating specific signalling pathways downstream of TLR2.

5.2.5 Exaggerated responses of *Irf7*$^{-/-}$ cDCs to TLR2 stimulation occur in the presence of wildtype cDCs and exogenous IFNα

In order to further clarify the mechanism behind the exaggerated activation profile seen with B6.*Irf7*$^{-/-}$ cDCs after TLR2 stimulation, components of the response to TLR stimulation that may be absent when cells lack IRF7 were supplemented into cDC activation cultures. To determine whether factors produced by IRF7-sufficient cDCs after TLR2 stimulation may affect the activation of *Irf7*$^{-/-}$ cDCs, CD11c^{hi} cells were sorted to high purity from spleens of B6J.CD45.1 (wildtype, IRF7-sufficient cells) and congenic (CD45.2) B6.*Irf7*$^{-/-}$ animals (**5.5A**). Sorted cDCs were then cultured at an approximately 50:50 ratio (**5.5B**) and stimulated with PAM_3CSK_4, as before. Gating on CD45.1 or CD45.2 during flow cytometric analysis allowed an assessment of activation, measured as the fold increase in CD86 expression over unstimulated cells, on wildtype and IRF7-deficient cDCs cultured in the same microenvironment. The presence of wildtype cDCs did not prevent the exaggerated expression of CD86 on cDCs after activation previously observed on IRF7-deficient cells (**5.5C**). Activation levels were

higher on cDCs lacking IRF7 at all time points post-stimulation, with significantly enhanced fold increases in CD86 expression of 20.38 ±0.01 on wildtype cDCs compared to 24.97 ±0.35 fold ($p<0.01$) on B6.*Irf7*$^{-/-}$ cells at 16hr p.s, in addition to increases of 31.76 ±0.79 fold compared to 38.02 ±0.02 ($p<0.01$) fold on wildtype and IRF7-deficient cDCs, respectively, at 24 hours post-stimulation.

IFNα is a major immune mediator which critically depends on IRF7 for its expression. As such, exogenous IFNα was added to cultures containing wildtype or IRF7-deficient cDCs to determine whether a lack of IFNα production in response to TLR2 stimulation was behind the exaggerated activation profile of B6.*Irf7*$^{-/-}$ cDCs.

Addition of exogenous IFNα did not prevent greater expression of CD80 on B6.*Irf7*$^{-/-}$ cDCs, with significantly enhanced fold increases in surface expression on these cells at all time points post-stimulation (**5.5D**). Wildtype cDCs showed modest fold increases in CD80 expression of 0.79 ±0.06, 1.09 ± 0.01, 2.04 ±0.06, 3.56 ±0.01 and 3.89 ±0.05 at 2, 4 8, 16 and 24 hours post-stimulation. This was in contrast to the more exaggerated expression of CD80 seen on B6.*Irf7*$^{-/-}$ cDCs, with increases of 1.38 ±0.01, 2.08 ±0.09, 3.64 ±0.01 and 6.84 ±0.27 fold (all $p<0.01$ compared to wildtype cDCs) at 2, 4, 8 and 16hr p.s, in addition to 8.46 ±0.05 fold ($p<0.001$) at 24hr post stimulation.

Exaggerated CD86 expression on cDCs lacking IRF7 was similarly unaffected by the presence of exogenous IFNα, with higher fold increases in CD80 expression on these cells at all time points, although only significantly so at 4, 16 and 24hr post-stimulation (**5.5E**). Unlike CD80 expression, the fold increases in CD86 upregulation on cDCs from both strains were higher in the presence of IFNα, at approximately twice that in its

absence. At 4hr post-stimulation, wildtype cDCs showed a 10.23 ±0.11 fold increase in CD86, compared to a 12.86 ±0.11 ($p<0.01$) fold increase on IRF7-deficient cells. CD86 expression was increased 38.61 ±2.19 and 45.77 ±4.79 fold at 16 and 24hr post-stimulation, compared with 52.13 ±1.88 and 68.44 ±3.87 (both $p<0.05$) fold increases on B6.*Irf7*$^{-/-}$ cDCs at these time points.

Similarly, the exaggerated expression of MHCII previously observed after stimulation of IRF7-deficient cDCs with PAM_3CSK_4 was not prevented by exogenous IFNα (**5.5F**). B6.*Irf7*$^{-/-}$ cDCs in the presence of IFNα showed significantly enhanced upregulation of surface MHCII compared to wildtype cDCs at all time points post-stimulation. Wildtype cDCs showed 1.44 ±0.02, 1.63 ±0.01, 2.48 ±0.05, 2.39 ±0.02 and 2.06 ±0.08 fold increases in expression of surface MHCII at 2, 4, 8, 16 and 24 hours post-stimulation. cDCs isolated from IRF7-deficient mice showed significantly higher fold increases in surface MHCII expression, at 2.26 ±0.06 ($p<0.01$), 2.78 ±0.01 ($p<0.001$), 4.20 ±0.01 ($p<0.01$), 4.95 ±0.02($p<0.001$) and 4.89 ±0.01 ($p<0.001$) fold at the respective time points post-stimulation.

Therefore, addition of either wildtype cDCs or exogenous IFNα was unable to prevent the exaggerated activation profile of B6.*Irf7*$^{-/-}$ cDCs in response to TLR2 stimulation, suggesting that IRF7 may be a component of the regulatory network activated after TLR stimulation in splenic cDCs.

5.2.6 Cytokine production by IRF7-deficient cDCs *in vitro*

$CD11c^{hi}MHCII^{hi}$ splenic cDCs were sorted from naïve C57BL/6 and B6.*Irf7*$^{-/-}$ mice and cultured for 24 hours in the presence of LPS or PAM_3CSK_4 with or without exogenous IFNα. Levels of IL-12p70 (**5.6A&C**) and IL-10 (**5.6B&D**) were determined in culture supernatant by ELISA. IL-12p70 production by cDCs in the absence of exogenous stimulation was impaired when cDCs lacked IRF7, represented by IL-12p70 levels of 13.7 ±1.21pg/ml in culture supernatant of wildtype cDCs and 4.94 ±0.80pg/ml ($p<0.01$) of cDCs isolated from B6.*Irf7*$^{-/-}$ mice. In response to stimulation with LPS, IRF7-deficiency severely impaired IL-12p70 production by cDCs, with wildtype cells producing 127.06 ±19.08 pg/ml after 24 hours and *Irf7*$^{-/-}$ cDCs producing 16.51 ±5.85pg/ml ($p<0.01$) after culture. The addition of exogenous IFNα did not alter this defective response, with wildtype cDCs producing 104.18 ±3.54 pg/ml of IL-12p70, compared to only 19.52 ±1.78 pg/ml ($p<0.001$) from IRF7-deficient cells after culture with both LPS + IFNα. Similarly impaired IL-12p70 production was observed in IRF7-deficient cells cultured with PAM_3CSK_4, with IL-12p70 at 83.72 ±11.60pg/ml in culture supernatant from wildtype cells and 19.84 ±7.51pg/ml ($p<0.01$) from cDCs lacking IRF7. Again, exogenous IFNα did not affect this deficiency, with wildtype cDCs producing 107.51 ±9.84pg/ml of IL-12p70 in the presence of both PAM_3CSK_4 and IFNα, and culture supernatants of cDCs isolated from B6.*Irf7*$^{-/-}$ mice only containing 21.33 ±3.31pg/ml ($p<0.01$).

In contrast to the defective IL-12p70 production by IRF7-deficient cDCs, IL-10 levels in culture supernatants of cDCs isolated from B6.*Irf7*$^{-/-}$ mice contained significantly higher levels of IL-10 when stimulated with either LPS or PAM_3CSK_4 (**5.6B&D**). This was represented by an increase in IL-10 production from 233.26 ±9.80pg/ml by

wildtype cDCs to 355.85 ±17.52pg/ml ($p<0.01$) by *Irf7*$^{-/-}$ cDCs after culture with LPS and from 534.56 ±10.97pg/ml to 871.53 ±8.67pg/ml ($p<0.01$) after culture with PAM_3CSK_4. Exogenous IFNα did not affect this altered cytokine production, with wildtype cDCs producing significantly less IL-10 in response to culture with both LPS and IFNα, at 211.94 ±22.13pg/ml from wildtype cDCs and 379.76 ±15.89pg/ml ($p<0.001$) from cDCs deficient in IRF7. Similarly, culture in the presence of both PAM_3CSK_4 and IFNα did not affect the differential production of IL-10, with wildtype cDCs producing 508.47 ±20.09pg/ml and IRF7-deficient cells producing 846.42 ±28.53pg/ml ($p<0.001$). Therefore, a lack of IRF7 in splenic cDCs leads to impaired IL-12p70 and enhanced IL-10 production in response to TLR2 and TLR4 ligands *in vitro*.

5.2.7 Acute infection with *Leishmania donovani* in IRF7-deficient mice

To address the potential relevance of the *in vitro* observations, a model of acute infection was employed in order to assess the impact of IRF7-deficiency on CD11c^{hi} dendritic cell activation in response to *in vivo* pathogen encounter. As such, C57BL/6 and B6.*Irf7*$^{-/-}$ mice were infected i.v. with a high dose of *L. donovani* amastigotes and infection allowed to establish for 24 hours. Mice were killed at this time point and spleens isolated. Spleen mass of both strains was increased as a result of infection, from 0.10 ±0.01mg in naïve C57BL/6 mice to 0.14 ±0.01 at 24hr post-infection (**5.7A**). Spleens of B6.*Irf7*$^{-/-}$ animals also increased in mass, from 0.11 ±0.01mg in naïve animals to 0.18 ±0.02 ($p<0.05$) by 24 hours of infection. Although slightly larger in infected B6.*Irf7*$^{-/-}$ animals, spleens were not significantly increased in size compared to infected wildtype mice. Splenic parasite burdens at 24 hours post-infection were determined by flow cytometry, and were not significantly altered as a result of IRF7-

deficiency (**5.7B**). Spleens of C57BL/6 mice contained $2.29x10^6$ $\pm 3.37x10^5$ parasites, whereas IRF7-deficient animals contained $2.39x10^6$ $\pm 9.57x10^5$ amastigotes.

In agreement with previous observations, splenic $CD11c^{hi}MHCII^{hi}$ cells were at an equivalent frequency of 1.06 ±0.08% and 1.06 ±0.07% in naïve wildtype and B6.*Irf7*$^{-/-}$ mice, respectively (**5.7C**). Upon infection, the proportion of these cells amongst total splenocytes significantly decreased, to 0.48 ±0.07% and 0.40 ±0.03% (both $p<0.01$) by 24 hours post-infection.

cDC activation as a result of acute infection with *L. donovani* was assessed by flow cytometry on $CD11c^{hi}MHCII^{hi}$ cells from C57BL/6 and B6.*Irf7*$^{-/-}$ mice, with activation levels expressed as the fold increase in surface costimulatory molecule expression on splenic cDCs from 24-hour infected mice, compared to the same cells from uninfected mice of the respective strain (**5.7D**). Acute infection with *L. donovani* led to modest increases in costimulatory molecule expression on cDCs from both strains of mice. Although not significantly so, CD40 upregulation on cDCs was impaired in infected IRF7-deficient mice, with CD40 expression increased 1.92 ±0.32 fold on wildtype cDCs, compared to 1.35 ±0.17 fold on IRF7 deficient cells. CD80 expression was barely enhanced as a result of infection in both strains, with C57BL/6 cDCs upregulating surface CD80 by 1.09 ±0.41 fold and B6.*Irf7*$^{-/-}$ cells showing a slight decrease in expression of 0.78 ±0.14 fold, relative to naïve cDCs of this genotype. Changes in surface CD86 expression were broadly equivalent in both strains, at 1.46 ±0.53 fold on wildtype cells and 1.57 ±0.22 fold on IRF7-deficient cDCs. Therefore, *in vivo* activation of cDCs as a result of acute *L. donovani* infection was broadly equivalent in the presence or absence of IRF7.

5.2.8 Global IRF7 deficiency limits cDC activation in response to TLR stimulation *in vivo*

To directly assess the contribution of IRF7 to the regulation of cDC activation in response to defined TLR ligands *in vivo*, C57BL/6 and B6.*Irf7*$^{-/-}$ mice were injected intravenously with TLR agonists and splenic cDC activation determined 24 hours later by flow cytometry (5.8). Splenic CD11c^{hi}MHCII^{hi} cDCs (**5.8A**) from both strains of mice were assessed for expression of CD80 and CD86 after intravenous injection of PBS or LPS (**5.8B**). Activation levels of cDCs from both strains were expressed as the fold increase in levels of surface CD80 or CD86 on cDCs from spleens of LPS-injected mice, compared to spleens of mice receiving PBS i.v. (**5.8C**). cDCs from wildtype mice showed a 2.34 ±0.31 fold increase in surface CD80, alongside a 4.88 ±0.76 fold increase in surface CD86 after LPS injection, whereas cDCs from B6.*Irf7*$^{-/-}$ mice receiving LPS i.v. showed smaller increases in CD80 and CD86 expression of 1.86 ±0.06 fold and 2.91 ±0.42 fold, respectively, 24 hours post-injection. However, these differences in activation were not significant.

As TLR2 signalling appeared to be affected most profoundly by a lack of IRF7 *in vitro*, PBS or PAM_3CSK_4 were administered to C57BL/6 and B6.*Irf7*$^{-/-}$ mice and cDC activation assessed by flow cytometry as before. Similar to that observed after injection of LPS, as well as *in vitro*, expression of CD86 occurred with a biphasic pattern, whereby a proportion of cDCs expressed high levels of costimulatory molecules when compared to cDCs from PBS-injected animals (**5.8D**). There was no significant difference in the fold increase in surface expression of CD80 on cDCs after PAM_3CSK_4 administration, at 2.36 ±0.08 fold and 2.35 ±0.13 fold on cDCs from wildtype or B6.*Irf7*$^{-/-}$ mice, respectively (**5.8E**). In contrast, upregulation of CD86 expression was

significantly impaired in B6.*Irf7*$^{-/-}$ mice, with wildtype cDCs showing a 6.04 ±0.18 fold increase in surface CD86 after PAM_3CSK_4 injection, compared with 4.37 ±0.28 fold ($p<0.01$) on cDCs from IRF7-deficient animals. In summary, *in vivo* administration of TLR agonists suggested that, in contrast to *in vitro* data, IRF7 may be required for optimum expression of this costimulatory molecule *in vivo*.

5.2.9 *In vivo* administration of PAM_3CSK_4 to microchimeric mice reveals enhanced CD86 expression on IRF7-deficient cDCs

Although useful in determining the systemic function of particular genes, mice with a global deficiency in immune-related genes may not provide a full complement of signals required for effective immune cell activation. Therefore microchimeric mice, in which a minority of hematopoietic cells of the desired genotype develop within an wildtype host, were used for *in vivo* inflammation studies to address the role of IRF7 in cDC activation in response to TLR2 signalling (**5.9**). B6J.CD45.1 mice were used as recipient animals and injected i.p. with Busulfan, before resting overnight and receiving bone marrow cells intravenously from congenic donor mice of the desired genotype; either C57BL/6 (wildtype) or B6.*Irf7*$^{-/-}$ (**5.9A**). After leaving to engraft for a period of 7-14 days, microchimeric mice were injected with PBS or PAM_3CSK_4 i.v., and dendritic cells assessed for expression of CD80 and CD86 by flow cytometry.

Spleens of microchimeric mice contained $CD11c^{hi}MHCII^{hi}$ cDCs derived from both host (CD45.1) and donor (CD45.2) genotypes (**5.9B**). There was no difference in the ability of C57BL/6 or B6.*Irf7*$^{-/-}$ bone marrow to engraft, with equivalent frequencies of endogenous vs chimeric cDCs regardless of which strain of donor mice was used. By

comparing surface expression of CD80 and CD86 on endogenous and chimeric cDCs after either PBS or PAM_3CSK_4 injection, levels of activation could be determined by expressing the fold change of CD80 (**5.9C**) or CD86 (**5.9D**) expression on chimeric cDCs as a result of TLR2 stimulation *in vivo*. There was no significant difference in fold upregulation of CD80 by endogenous cDCs compared to wildtype chimeric cDCs, at 2.33 ±0.10 fold and 2.45 ±0.32 fold, respectively. Comparable upregulation of CD80 was also seen when endogenous cDCs were compared to IRF7-deficient chimeric cDCs, with 2.61 ±0.11 and 2.54 ±0.10 fold increases in CD80 expression on the respective cDC populations from spleens of mice 24 hours after PAM_3CSK_4 injection.

CD86 upregulation after PAM_3CSK_4 administration was also comparable on endogenous cDCs when compared to C57BL/6 chimeric cDCs, at 3.54 ±0.47 and 3.51 ±0.70 fold, respectively, when compared with cDCs of either population in spleens of PBS-treated mice. In contrast, a lack of IRF7 in chimeric cDCs led to significantly exaggerated expression of CD86 after TLR2 stimulation, with these cells increasing surface CD86 expression 5.47 ±0.18 fold by 24 hours, compared with 4.08 ±0.11 fold ($p<0.01$) on endogenous cDCs in these microchimeric mice.

In summary, a lack of IRF7 expression by cDCs stimulated within an IRF7-sufficient environment led to exaggerated expression of CD86 and so suggests that, in the presence of an intact immune environment, cDCs lacking IRF7 are not capable of effectively regulating CD86 expression in response to TLR2 stimulation *in vivo*.

5.3 Discussion

This study reveals a novel role for IRF7 in regulating TLR2-induced costimulatory molecule expression by splenic cDCs *in vitro* and *in vivo*. Unlike many other members of this transcription factor family, IRF7 is redundant with respect to the *in vivo* development of cDC subsets. However, a lack of IRF7 expression by cDCs results in exaggerated costimulatory molecule expression and altered cytokine producing potential *in vitro*. Although TLR ligand administration to mice with global IRF7-deficiency fails to recapitulate the *in vitro* data, administration of PAM_3CSK_4 to microchimeric mice indicates a key role for IRF7 in the regulation of CD86 expression by splenic cDCs *in vivo*.

IRF7 appears to play a limited role in the development of cDC subsets and other splenic immune cell types, supporting previous data showing normal cell distribution in the spleens of B6.*Irf7*$^{-/-}$ mice by immunohistochemistry [502] and in the livers of IRF7-deficient mice by flow cytometry (Beattie *et al,* in press). However this is in stark contrast to mice lacking expression of other IRF family members, with normal cDC (cf. above), CD8α^+ T cell [638, 639] and NK cell [640-642] development relying on the expression of several IRFs, suggesting either that compensatory mechanisms for immune cell development exist in the absence of IRF7, or that the functions of this transcription factor are more restricted than other members of the same family.

As IRF7 is critical for the optimal induction of type I IFN expression, it was surprising that a deficiency in this transcription factor led to alterations in the response of cDCs to ligation of TLR2; signalling through which was, until recently, not thought to lead to

type I IFN expression [643]. However it is now becoming clear that TLR2 signalling can lead to type I IFN production under some conditions. This was first observed during the response of mice to vaccinia virus, where a subset of $Ly6C^{hi}$ inflammatory monocytes produced type I IFN in an IRF3 and IRF7-dependent manner after TLR2-mediated recognition of viral ligands [644]. This was proposed to be restricted to both inflammatory monocytes and virus-derived stimuli. However, more recent studies have revealed a capacity for type I IFN production in other cell types and in response to diverse TLR2 ligands, including PAM_3CSK_4 [637, 645]. This is thought to be dependent upon the localisation of TLR2 to endolysosomal membranes, with subsequent signalling from this compartment leading to IFNα/β production- more akin to the mechanisms employed by TLR3, TLR7 and TLR9 [646, 647]. The location of TLR2 to endosomal compartments and subsequent IFNα/β production may, as with TLR4, be dependent upon TRAF3, as forced localisation of TRAF3 to the plasma membrane enables IFNβ production after PAM_3CSK_4 stimulation, rather than the classical plasma membrane-restricted signalling that initiates pro-inflammatory cytokine production in response to TLR2 [648]. However it is still unclear as to the precise molecular mechanisms leading to TLR2-induced IFNα/β production.

Although IFNα production is critically dependent upon IRF7, IFNβ production in response to LPS stimulation occurs at normal levels in IRF7-deficient cells [547]. However, the recent studies showing type I IFN production in response to endosomal TLR2 signalling all revealed a key requirement for IRF7 in the induction of IFNβ [637, 645, 649]. Therefore, in addition to a lack of IFNα, cDCs stimulated with TLR2 ligands would also presumably lack IFNβ production. Although IFNβ has been shown to be required for maximal costimulatory molecule expression on peritoneal macrophages in

response to LPS stimulation [622], splenic cDCs which are deficient in the IFNαβ receptor (IFNαβR) and thus cannot respond to either type I IFN, show greatly enhanced expression of CD80 and CD86 in response to infection with *Listeria monocytogenes in vivo* [650]. As the data presented here show that IRF7 deficiency led to exaggerated co-stimulatory molecule expression on cDCs after TLR2 but not TLR4 signalling and exogenous IFNα could not prevent this occurring, this allows for a hypothetical model to be generated by which the TLR-mediated induction of IFNβ can, at least in splenic cDCs, also contribute to the regulation of co-stimulatory molecule expression under certain conditions (**5.10**). The effects of exogenous IFNβ on the activation of *Irf7*$^{-/-}$ cDCs stimulated with TLR2 ligands *in vitro* would allow this to be tested, as would the stimulation of cDCs lacking the IFNβ receptor *in vitro* or *in vivo*.

Although IFNβ may provide some explanation as to the impaired regulation of costimulatory molecule expression, as PAM_3CSK_4-stimulated IRF7-deficient cDCs from cultures containing a 50:50 mix of wildtype cDCs also showed exaggerated CD86 expression, it is possible that a type I IFN independent mechanism may also contribute to these observations. Tripartite motif-containing (TRIM) proteins comprise a family of type I IFN-inducible factors with diverse roles in the immune system [651]. Of particular relevance is TRIM30α, recently found to contain a putative IRF7 binding sequence in its promoter region (Phillips, unpublished). This protein acts to restrict NF-κB –mediated signalling downstream of TLR ligation by interacting with TAK1 and degrading TAB2 and TAB3 [195]. Interestingly TAB2 itself also contains a putative IRF7-binding element (Phillips, unpublished), suggesting that IRF7 may be capable of regulating the activation of NF-κB directly and through the actions of TRIM30α. As enhanced costimulatory molecule expression is dependent on NF-κB [652, 653], it is

possible that when this pathway is not correctly regulated, as may be the case in IRF7-deficient cDCs, that this results in a failure to regulate CD86 expression and thus leads to enhanced accumulation of this costimulatory molecule on cDCs. Further work will be required to confirm whether IRF7 does directly interact with TRIM30α, perhaps by chromatin immunoprecipitation assays, and to provide molecular evidence for the hypothetical mechanism described.

The altered cytokine profile of cDCs lacking IRF7 and stimulated *in vitro* was interesting, as it appeared to show uncoupling of costimulatory molecule expression and cytokine production in response to TLR stimulation. In particular, the greatly reduced production of IL-12p70 by IRF7-deficient cDCs in response to TLR2 signalling was surprising, given the exaggerated expression of costimulatory molecules occurring simultaneously on these cells. This was unexpected, as previous reports have described exaggerated inflammatory cytokine production in the absence of IRF7, with MCMV infected B6.*Irf7*$^{-/-}$ mice having significantly elevated levels of serum IL-12p70 compared to wildtype animals, associated with enhanced IFNγ production but only moderate changes in host pathology [546]. Exacerbated pro-inflammatory cytokine production has also been reported in IRF7-deficient mice in a model of liver pathology; although this appears to be due to a failure in the IFNα-mediated induction of soluble IL-1Rα rather than any direct regulation of cytokine gene expression by IRF7 [649]. However, these studies did not address cytokine production from defined cell populations, and so the enhanced levels of systemic cytokines could be due to differential responses of distinct immune cell populations when deficient in IRF7.

In light of the reduced IL-12p70 production by IRF7-deficient cDCs in response to both TLR2 and TLR4 signalling, a potential mechanism for this observed defect is suggested by the reported requirement for cooperation between IRF1 and IRF8 in order to generate maximal IL-12 production by APCs [550, 552, 616-619, 654]. As IRF7 has been shown to interact with IRF8 [655] and is predicted to bind both IRF1 and IRF8 [629, 656], it is possible that in the absence of IRF7, full induction of IRF1 and IRF8 expression does not occur and thus IL-12 production is impaired. This is also supported by data showing that TRIM21, a putative target of IRF7 (Phillips, unpublished), is required for the ubiquitination of IRF8 and maximal IL-12 gene expression [657]. As such, it is feasible that IRF7 is required for full IL-12 production via the direct and indirect activation of other IRFs.

Alongside their defective production of IL-12p70, cDCs deficient in IRF7 produced elevated levels of IL-10 in response to TLR ligation. This is similar to IRF1-deficient splenic DCs, which show impaired IL-12 and enhanced IL-10 production *in vitro* [615]. However, splenic DCs from *Irf1*$^{-/-}$ mice have limited upregulation of costimulatory molecule expression in response to TLR ligation: a phenotype distinct from PAM_3CSK_4-stimulated splenic cDCs lacking IRF7 and indicating that differential mechanisms underlie these broadly similar observations. It is possible that the enhanced IL-10 production by both IRF1 and IRF7-deficient cDCs underlies the impaired capacity for IL-12p70 production- similar to the autocrine regulation of IL-12 production by IL-10 discussed earlier in this study (cf. Chapter 3). Alternatively, as IL-10 suppresses costimulatory molecule expression by APCs [658-660], it is possible that the enhanced IL-10 expression by IRF7-deficient cDCs is part of a negative feedback mechanism aimed at limiting the exaggerated costimulatory molecule expression that occurs on these cells. However, as *Irf7*$^{-/-}$ cDCs stimulated with both LPS and

PAM_3CSK_4 showed enhanced IL-10 production, yet only those stimulated through TLR2 showed exaggerated costimulatory molecule expression, this potential mechanism is unlikely to be the sole reason for the enhanced IL-10 production by IRF7-deficient cDCs. Although no direct links between IRF7 and IL-10 production are known, IRF4 can directly modulate IL-10 gene expression (cf. Chapter 4 and [557, 558]) and so the direct regulation of IL-10 production is a possibility. As enhanced IL-10 production is also observed in IRF7-deficient hepatic (Beattie *et al*) and splenic (Chapter 4) $CD4^+$ T cells during inflammation *in vivo*, it is possible that IRF7 is playing a role in regulating the expression of this cytokine in a range of cell types.

Despite some ambiguity as to the receptor-ligand interactions involved, there is evidence that conserved elements of *Leishmania donovani* are recognised by TLR2 (cf. Chapter 3) and so infection with this pathogen was suitable to assess the impact of IRF7 deficiency on *in vivo* cDC activation in response to TLR2 signalling. However, in contrast to the *in vitro* data, splenic cDCs from mice with a global deficiciency in IRF7 did not display exaggerated expression of co-stimulatory molecule expression after infection *in vivo*. This is likely due to the requirement for type I IFN production for optimal co-stimulatory molecule expression, as detailed above. However in light of the complexities surrounding the recognition of *L. donovani* by PRRs, it was also possible that this model of inflammation was not eliciting a strong enough response, or did not provide enough available TLR ligands for optimal cDC activation. Therefore the injection of defined TLR ligands i.v. was carried out in order to attempt to deliver TLR ligands to splenic cDCs as efficiently as possible.

Systemic administration of TLR ligands led to similar observations as those seen after infection with *L. donovani*, with cDCs from C57BL/6 and B6.*Irf7*$^{-/-}$ mice having equivalent costimulatory molecule expression 24hrs after LPS injection *in vivo*. cDCs from both strains had equivalent expression of CD80 in response to PAM_3CSK_4 injection, although significantly reduced expression of CD86 when isolated from B6.*Irf7*$^{-/-}$ animals. This strongly suggested that in mice with a global IRF7 deficiency, optimal expression of CD86 did not occur due to a lack of IRF7-dependent immune mediators in these animals.

Although this could have been addressed by the injection of recombinant IFNα and/or IFNβ, other factors downstream of IRF7 signalling may have also been involved, such as IL-6 [629], so a different approach was taken. This took advantage of the capacity for Busulfan, an alkylating agent, to partially ablate the stem cell compartment in mice and allow for the generation of microchimeric animals bearing populations of donor-derived hematopoietic cells from congenic wildtype or IRF7-deficient backgrounds, which will develop in an intact, IRF7-sufficient microenvironment (Moore *et al* and [661, 662]). Injecting these mice with a TLR2 ligand revealed that CD86, but not CD80, expression was upregulated to a greater extent on IRF7-deficient cDCs *in vivo*; strongly implicating a role for IRF7-dependent factors in both the initial expression and subsequent regulation of costimulatory molecule expression. As CD86 is a key co-stimulatory molecule implicated in the generation of bystander activated T cells, such as occurs during infection with *L. donovani* [405], work is currently ongoing to determine whether a lack of IRF7 enhances the capacity for bystander activation of CD8α^{+} T cells *in vitro*.

In summary, this data reveals a novel role for IRF7 in the regulation of TLR2-induced costimulatory molecule expression by splenic cDCs *in vitro* and *in vivo*. This appears to be distinct from the regulation of pro-inflammatory cytokine production, as cDCs displaying exaggerated costimulatory molecule expression *in vitro* had a significantly impaired capacity for IL-12p70 production. In contrast, IL-10 production was enhanced, suggesting the potential for an autocrine negative feedback loop functioning to restrict this hyperactivation. Although the molecular mechanisms remain to be experimentally determined, preliminary data suggesting interactions between IRF7 and regulators of TLR-induced NF-κB expression provide a potential explanation as to the dysregulated costimulatory molecule expression observed. As regulating the expression of costimulatory molecules by cDCs is critical to the effective control of immune responses, further elucidating the pathways required for the efficient regulation of DC function will allow a greater understanding of DC biology, as well as revealing potential novel therapeutic targets for future development.

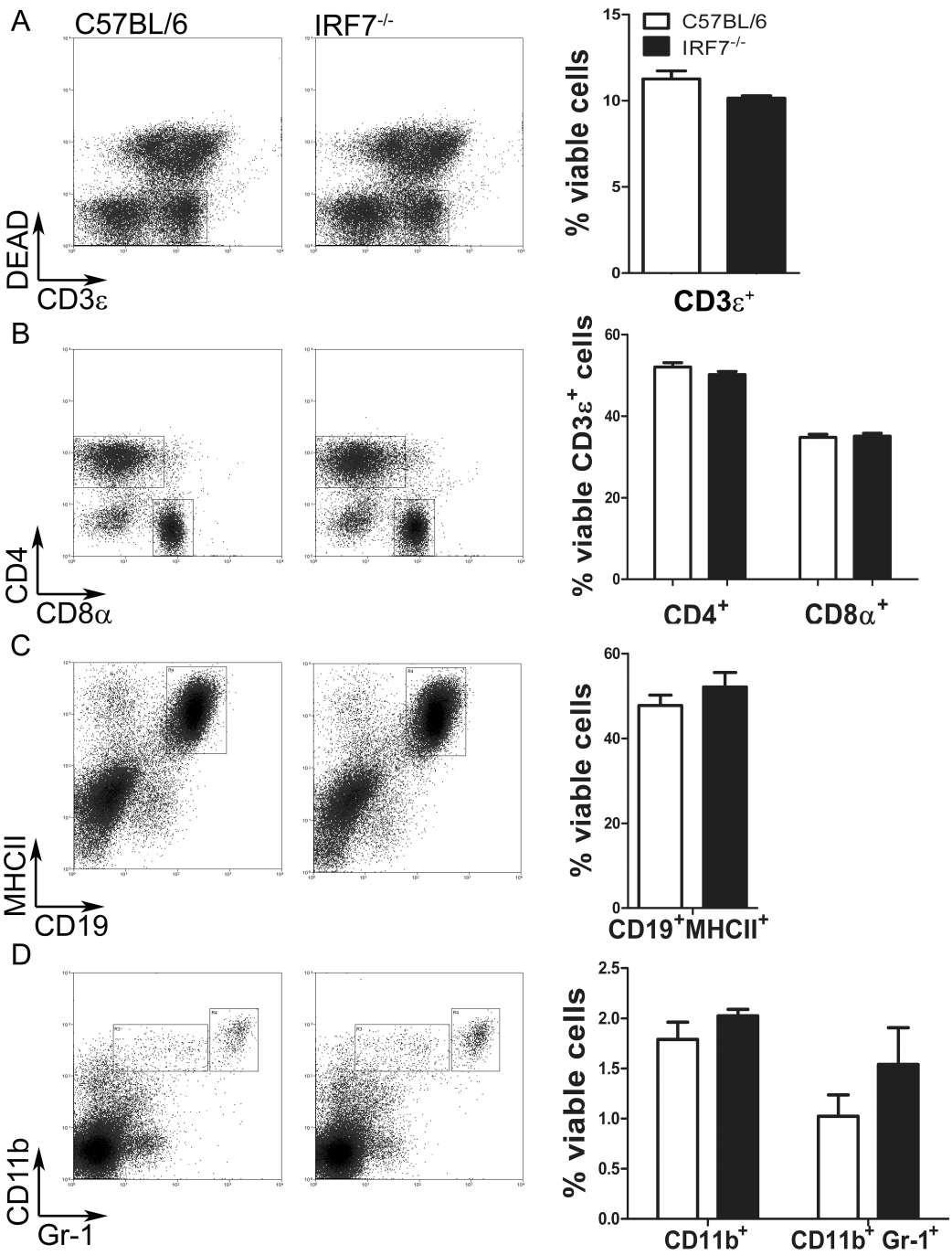

Figure 5.1 IRF7 deficiency does not affect spleen cell composition. The frequency of **A,** splenic $CD3\varepsilon^+$ T cells, **B,** $CD4^+$ and $CD8\alpha^+$ T cells, **C,** $CD19^+MHCII^+$ B cells and **D,** $CD11b^+$ monocytes and $CD11b^+Gr\text{-}1^+$ neutrophils were assessed by flow cytometry in steady state C57BL/6 (open bars) and B6.*Irf7*$^{-/-}$ (closed bars) mice. Data show representative flow plots and where quantified show the mean frequency ±SEM of indicated cell type in spleens of 4-5 mice per group. Representative of three separate experiments.

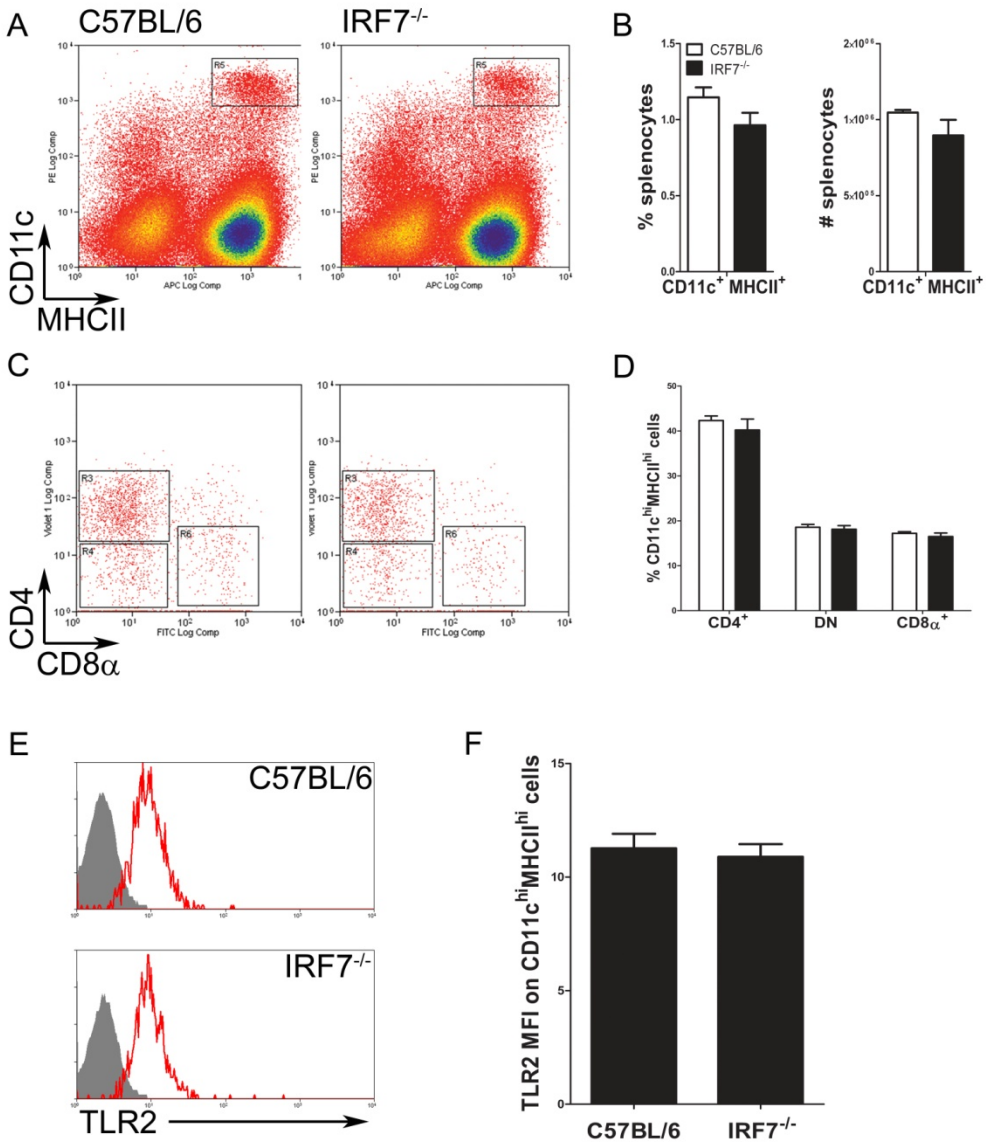

Figure 5.2 Splenic cDC subset development and TLR2 expression is independent of IRF7. The presence of splenic CD11c^{hi}MHCII^{hi} cDCs in C57BL/6 and B6.*Irf7*$^{-/-}$ mice was determined by flow cytometry (**A**&**B**). CD4^{+}, DN and CD8α^{+} cDC subset frequency was determined in the CD11c^{hi}MHCII^{hi} compartment of wildtype and IRF7-deficinet animals (**C**&**D**). Expression of TLR2 on CD11c^{hi}MHCII^{hi} cells from C57BL/6 and B6.*Irf7*$^{-/-}$ mice was determined by flow cytometry (**E**&**F**). Flow plots and histograms in **A**, **C** and **E** are representative, data in **B**, **D** and **F** show mean frequency ±SEM of indicated cell population in C57BL/6 (open bars) and B6.*Irf7*$^{-/-}$ (closed bars) mice. Histograms in **E** are gated on CD11c^{hi}MHCII^{hi} cells gated as in **A** and show specific TLR2 staining (open line) compared to isotype control antibody (filled histogram). Data are from n=4-5 mice per group and representative of three separate experiments.

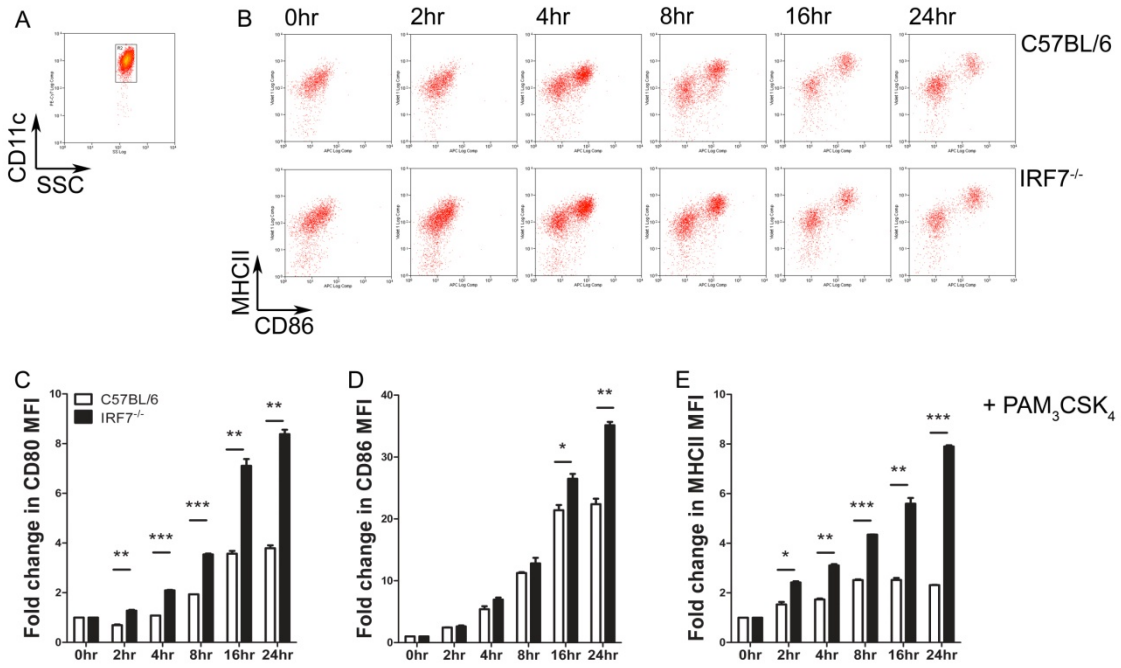

Figure 5.3 IRF7-deficient splenic cDCs are hyperresponsive to a TLR2 agonist *in vitro*. **A**. CD11c^{hi} cDCs were sorted to ~99% purity from the spleens of C57BL/6 and B6.*Irf7*$^{-/-}$ mice. Cells were cultured in triplicate at 1x10^6 cells/ml in the presence of 10µg/ml PAM_3CSK_4. At the indicated times post-stimulation, cells were removed and assessed by flow cytometry for expression of CD80, CD86 and MHCII. Flow plots showing progressive cDC activation in terms of CD86 and MHCII expression are shown in **B**, the fold increase in surface expression of CD80, CD86 and MHCII on cDCs at the indicated time point over unstimulated cDCs are shown in **C**, **D** and **E**, respectively. **A** and **B** show representative flow plots, **C**-**E** show mean fold increase ±SEM in surface expression of indicated proteins on cDCs from C57BL/6 (open bars) or B6.*Irf7*$^{-/-}$ (closed bars) mice, compared to unstimulated cDCs from the same strain. Data are pooled from three individual experiments. *=p<0.05 **= p<0.01, ***=p<0.001.

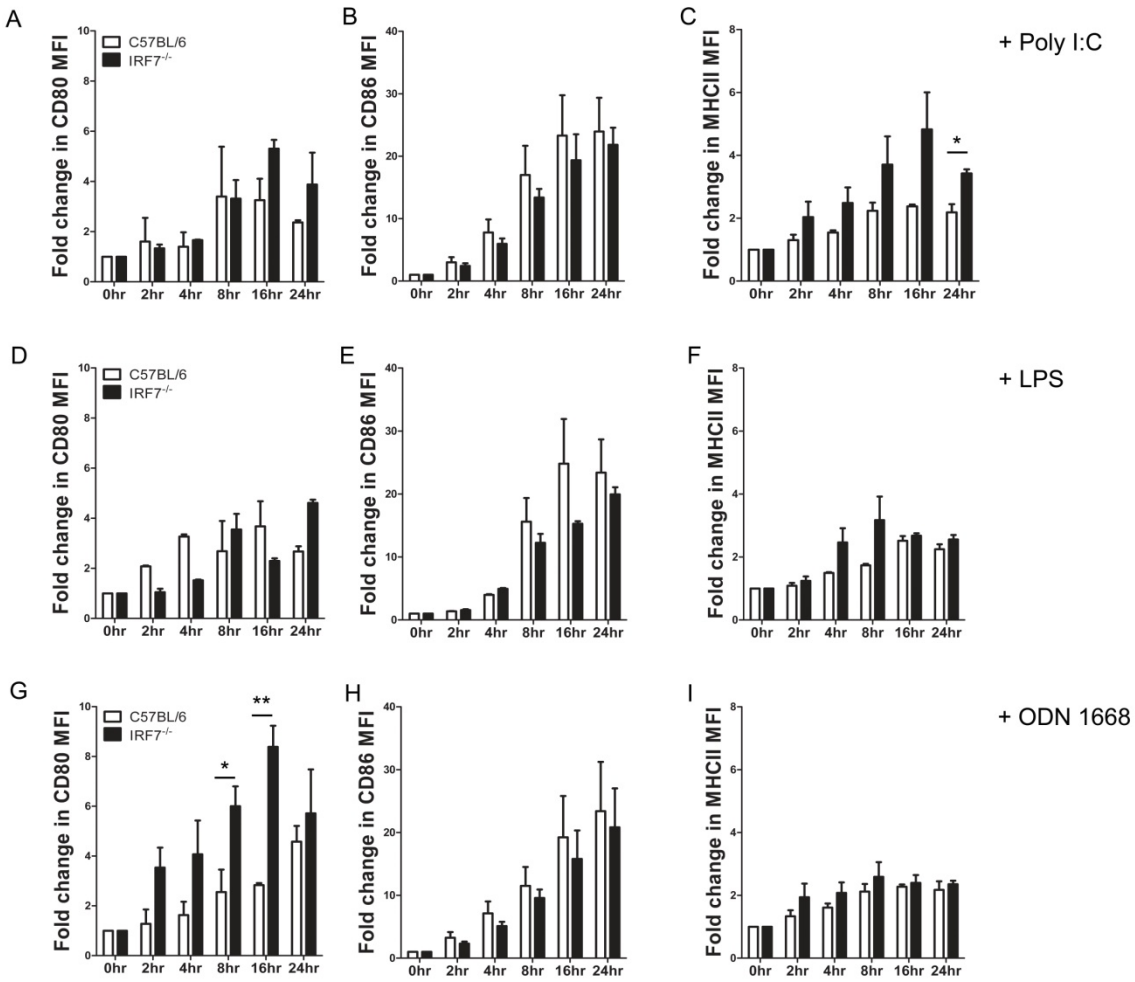

Figure 5.4 IRF7 deficiency has a limited impact on splenic cDC responses to TLR3, TLR4 and TLR9 signalling. Splenic CD11c^{hi} cDCs were sorted from C57BL/6 and B6.*Irf7*$^{-/-}$ mice as before. Cells were plated, in triplicate, and stimulated with 100µg/ml Poly (I:C), 1µg/ml LPS or 10µg/ml ODN:1668. At the indicated times post-stimulation, cells were removed and assessed by flow cytometry for their fold increase in expression of CD80 (**A**, **D**&**G**), CD86 (**B**, **E**&**H**) and MHCII (**C**, **F** and **I**) over unstimulated cDCs. Data show mean fold increase ±SEM in surface expression of indicated proteins on triplicate wells containing cDCs from C57BL/6 (open bars) or B6.*Irf7*$^{-/-}$ (closed bars) mice, compared to unstimulated cDCs from the same strain. Data are pooled from two individual experiments. *=$p<0.05$ **= $p<0.01$,

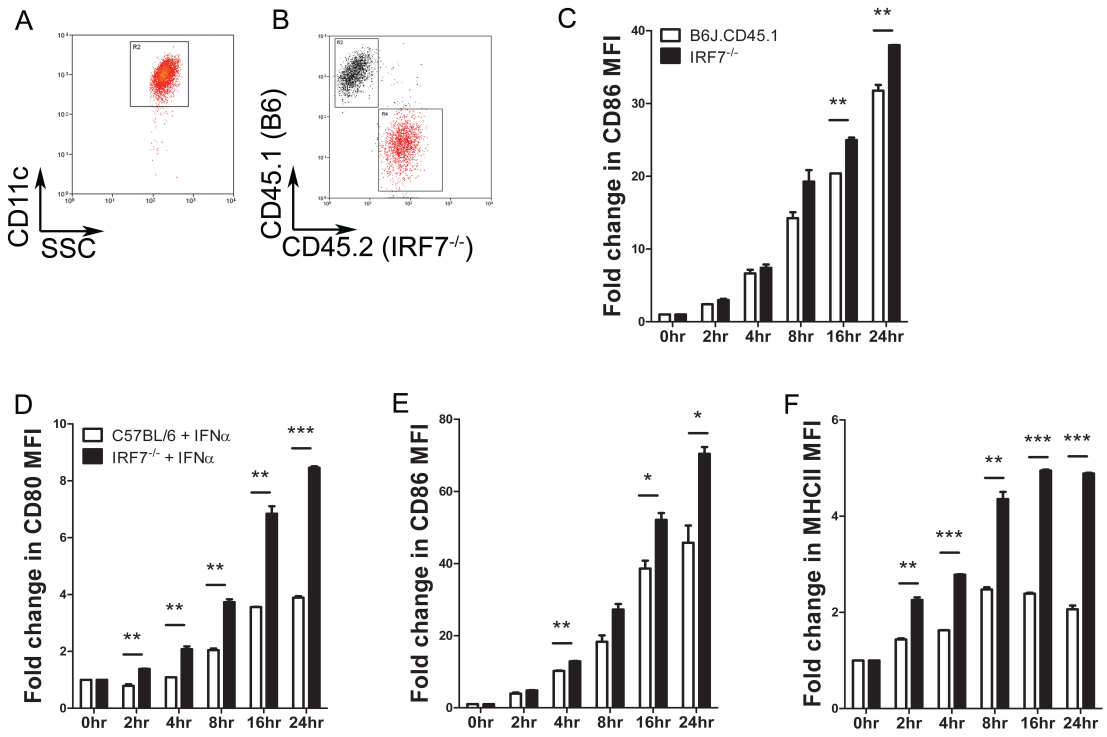

Figure 5.5 Exaggerated CD86 expression after TLR2 stimulation occurs in the presence of IRF7-sufficient cDCs and exogenous IFNα. **A**. Splenic CD11c^{hi} cDCs were sorted from C57BL/6, B6.*Irf7*$^{-/-}$ and congenic B6J.CD45.1 mice. **B**. cDCs from B6.*Irf7*$^{-/-}$ and B6J.CD45.1 mice were cultured at ~50:50 ratio to a final concentration of 1x10^6 cells/ml, in the presence of 10μg/ml PAM_3CSK_4. **C**. The fold increase in expression of CD86 on cDCs from either strain, in the same well, was determined by flow cytometry at the indicated times post-stimulation. **D-F**, C57BL/6 and *Irf7*$^{-/-}$ cDCs were cultured, in triplicate, in the presence of 10μg/ml PAM_3CSK_4 and 1000 U/ml IFNα. At the indicated times post-stimulation, cells were removed and assessed by flow cytometry for their fold increase in expression of CD80 (**D**), CD86 (**E**) and MHCII (**F**) over unstimulated cDCs. **A** and **B** show representative flow plots, **C-F** show mean fold increase ±SEM in surface expression of indicated proteins on cDCs from B6J.CD45.1 (open bars in **C**), C57BL/6 (open bars in **D-F**) or B6.*Irf7*$^{-/-}$ (closed bars) mice, compared to unstimulated cDCs from the same strain. Data are from one (**B-C**) or two (**D-F**) experiments. *=p<0.05 **= p<0.01, ***=p<0.001

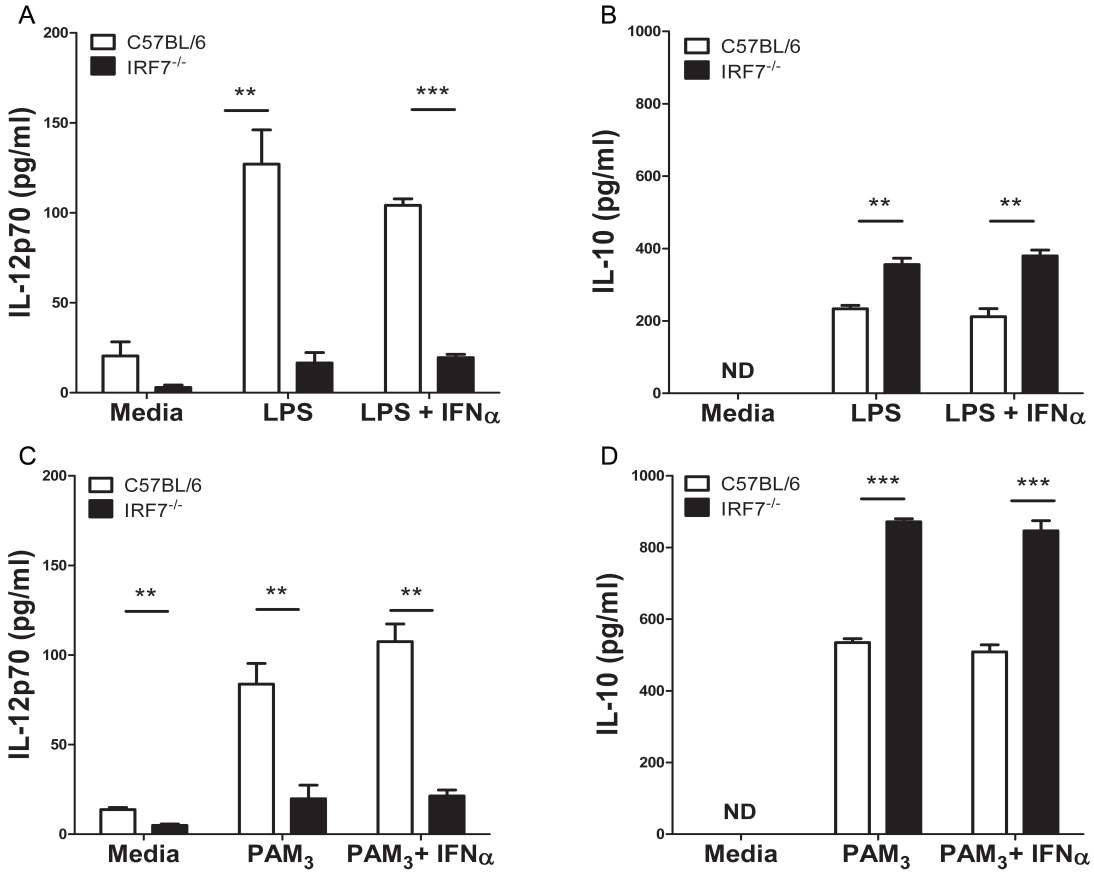

Figure 5.6 Cytokine production by IRF7-deficient cDCs *in vitro*. CD11c^{hi} cDCs were sorted from spleens of C57BL/6 and B6.*Irf7*$^{-/-}$ mice and cultured in triplicate for 24 hours in the presence of 10µg/ml PAM_3CSK_4, 1µg/ml LPS and 1000U/ml IFNα, or combinations thereof. After culture, supernatants were assessed by ELISA for presence of IL-12p70 (**A**&**C**) or IL-10 (**B**&**D**). Data show mean concentration of cytokine from triplicate wells ±SEM and are representative of two experiments. **= $p<0.01$ *=$p<0.001$

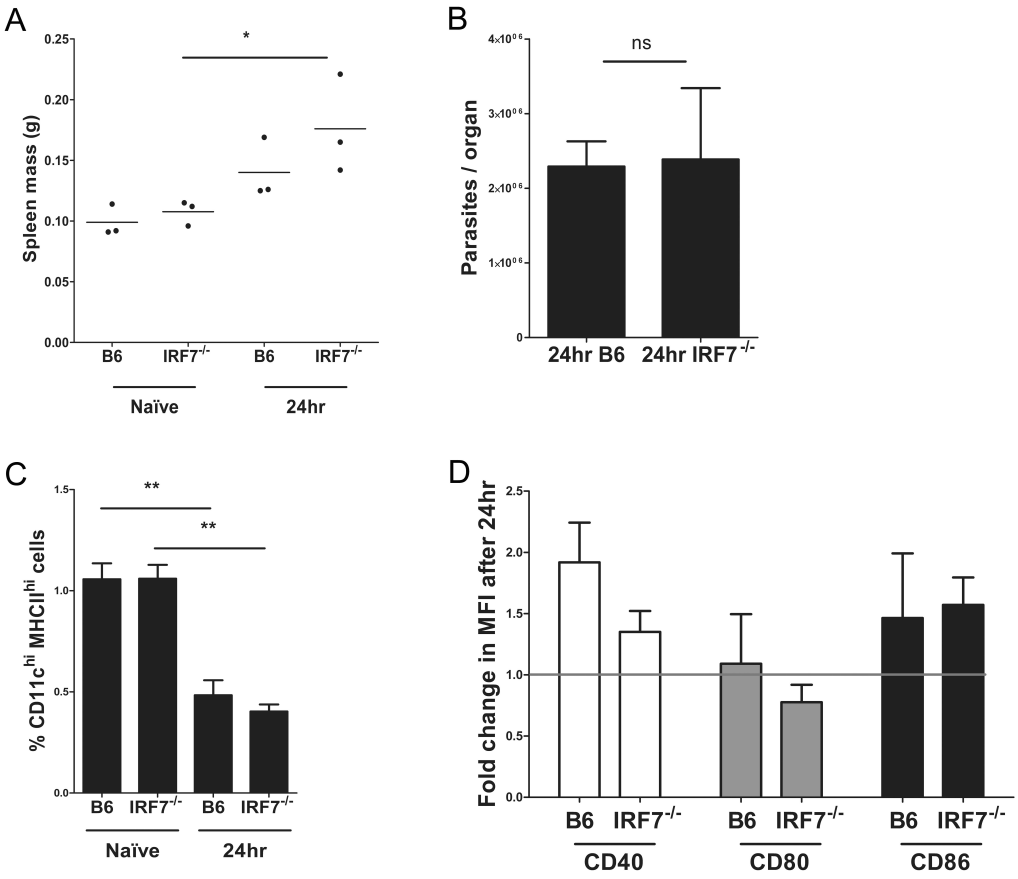

Figure 5.7 Acute infection with Leishmania donovani in IRF7-deficient mice. C57BL/6 and B6.*Irf7*$^{-/-}$ mice were infected via the lateral tail vein with a high dose ($4.5x10^7$) of *L. donovani*. **A**. At 24hr post infection, spleen mass in infected and naïve control animals was determined. **B**. Parasite burden in spleens of infected mice were determined by flow cytometry. **C**. The frequency of splenic $CD11c^{hi}MHCII^{hi}$ cDCs in naïve and infected mice was assessed by flow cytometry. **D**. Activation levels of cDCs as a result of acute infection were determined by quantifying the fold change in MFI of surface CD40, CD80 and CD86 on cDCs from infected mice compared with those from naïve animals. Data are mean ±SEM (**B**-**D**) and from one experiment with three mice per group. *=$p<0.05$ **= $p<0.01$

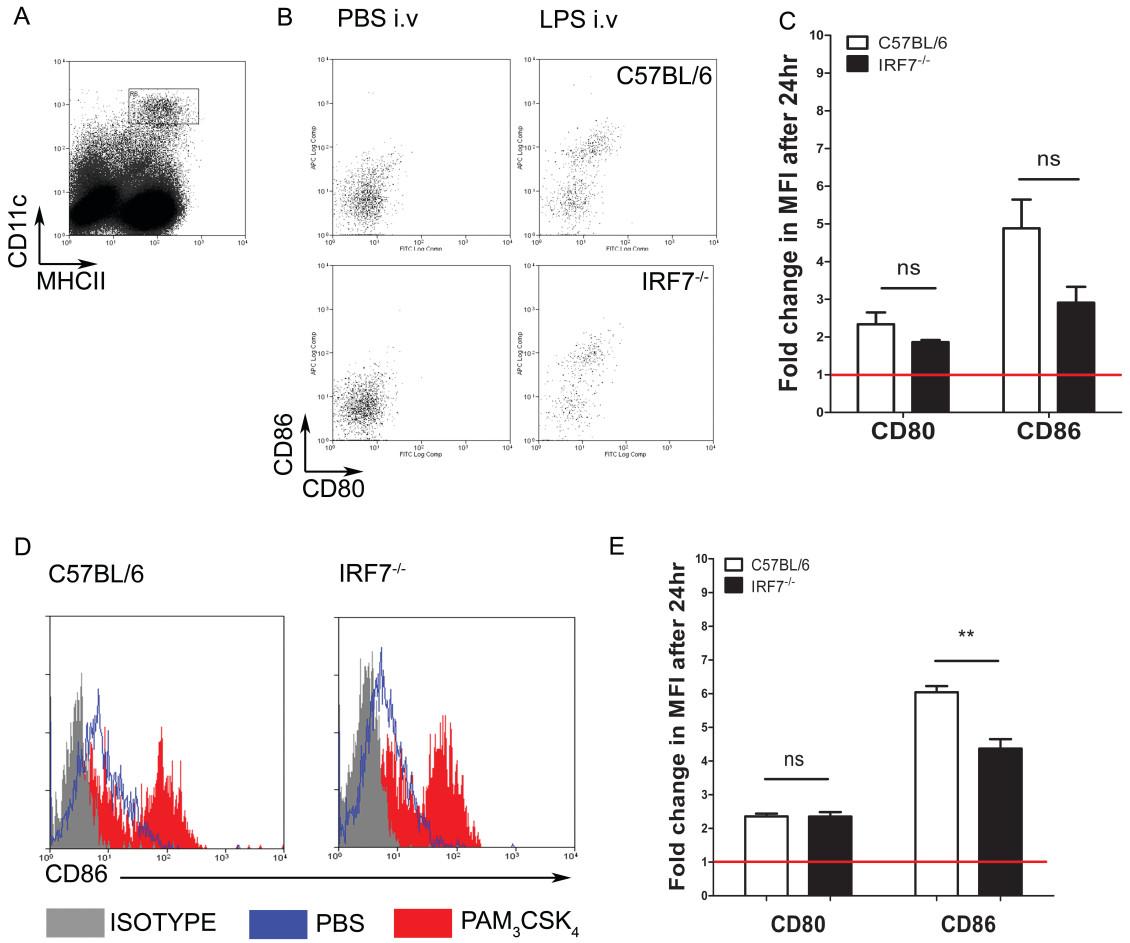

Figure 5.8 Global IRF7 deficiency limits cDC activation in response to TLR stimulation *in vivo*. A-C. C57BL/6 and B6.*Irf7*$^{-/-}$ mice received intravenous injections of 5µg/mouse LPS or **D&E**, 5µg/mouse PAM_3CSK_4 in 200µl PBS, or 200µl PBS alone. After 24 hours, activation of splenic CD11c^{hi}MHCII^{hi} cDCs (shown in **A**) after LPS (**B&C**) or PAM_3CSK_4 (**D&E**) administration *in vivo* was assessed by quantifying changes in surface expression of CD80 and CD86 by flow cytometry. Flow plots and histograms in **A**, **B** and **D** are representative, data in **C** and **E** show the mean fold change in indicated surface protein ±SEM on cDCs from C57BL/6 (open bars) and B6.*Irf7*$^{-/-}$ (closed bars) mice after TLR agonist injection compared to cDCs from mice receiving PBS only. Data are from one (**A-C**) or representative of two (**D&E**) experiments with 3 or 4 mice per group. **= $p<0.01$

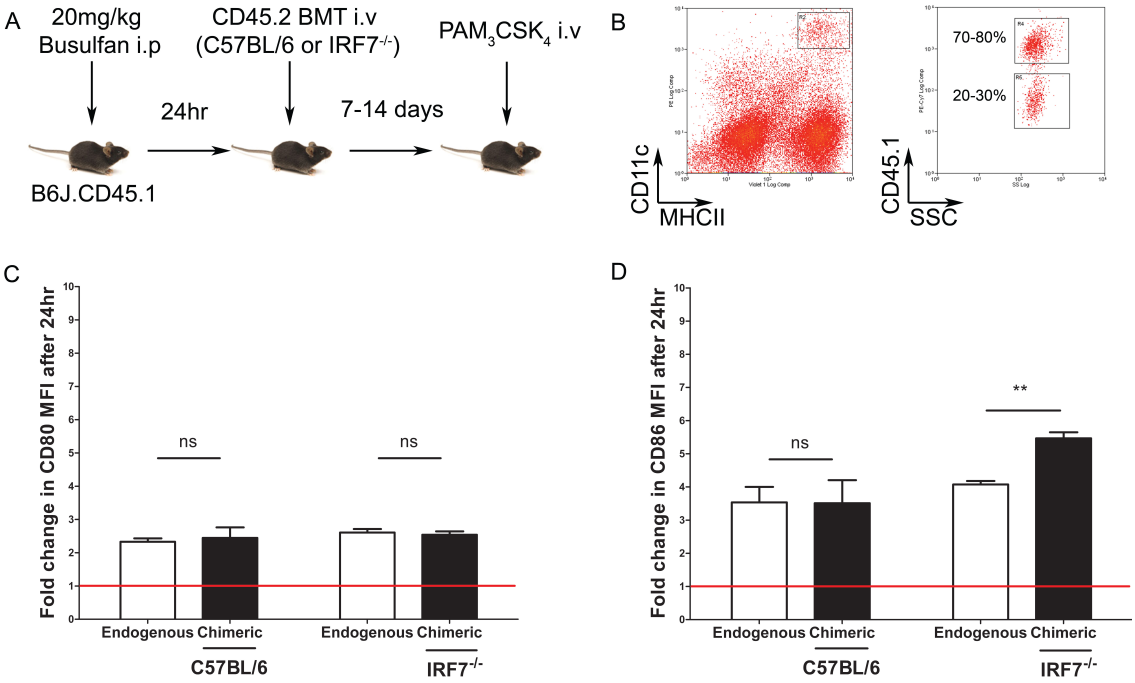

Figure 5.9 In vivo administration of PAM_3CSK_4 to microchimeric mice reveals enhanced CD86 expression on IRF7-deficient cDCs. B6.*Irf7*$^{-/-}$ or C57BL/6 microchimeric mice were generated by bone marrow transfer into Busulfan-treated congenic B6J.CD45.1 hosts (cf. Chapter 2). **A**. 7 to 14 days post-engraftment, microchimeric mice bearing *Irf7*$^{-/-}$ or C57BL/6 chimeric cell populations received 5µg/mouse PAM_3CSK_4 intravenously (**A**). Splenic CD11c^{hi}MHCII^{hi} cDC compartments in microchimeric animals were comprised of endogenous (CD45.1^{+}) and chimeric (CD45.1^{-}) cell populations (**B**). After 24 hours, activation of splenic cDCs after PAM_3CSK_4 administration *in vivo* was assessed by quantifying changes in surface expression of CD80 (**C**) and CD86 (**D**) by flow cytometry. Flow plots in **B** are representative, data in **C** and **D** show the mean fold change in indicated surface protein ±SEM on cDCs from the indicated endogenous or chimeric cell compartments in microchimeric mice after PAM_3CSK_4 injection (closed bars) compared to cDCs from mice receiving PBS only (open bars). **= $p<0.01$

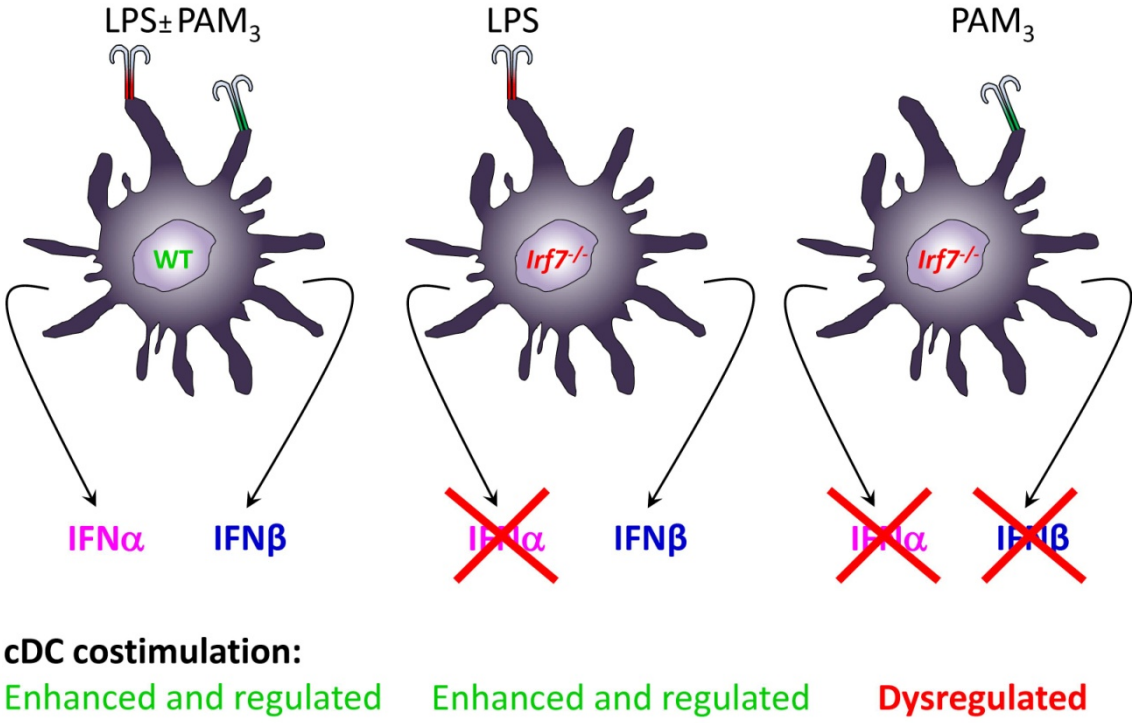

Figure 5.10 Hypothetical model for the differential impact of IRF7-deficiency on TLR2 and TLR4-mediated activation of cDCs. In wildtype cDCs, ligation of TLR2 and TLR4 leads to efficient induction of IFNα and IFNβ and allows for the regulated expression of costimulatory molecules. When cDCs deficient in IRF7 are stimulated with a TLR4 ligand, IFNα induction is abolished, but IFNβ expression is intact. This allows for the regulated expression of costimulatory molecules on cDCs. When IRF7-deficient cDCs are stimulated with a TLR2 ligand, production of both IFNα and IFNβ is abolished and this leads to the dysregulated expression of costimulatory molecules.

Chapter 6: Concluding discussion

Although DCs have a fundamental role in the generation of effective antigen-specific immune responses [9], it is now clear that they also significantly contribute to the regulation of immunity [124]. However, the majority of previous studies have focussed on maintaining tolerance to tissue associated antigen or examined the regulatory role of immature, *in vitro* derived or non-conventional DC populations during infection. This study demonstrates for the first time that $CD11c^{hi}$ cDCs are major negative regulators of immune responses to pathogens *in vivo*, highlighting their functional plasticity and revealing a novel, paradoxical capacity for these potent APCs to inhibit effective immunity to the protozoan parasite, *Leishmania donovani*.

The identification of a regulatory cytokine network operating at the level of the cDC extends our knowledge of the profound alterations in APC function during chronic infection. Impaired production of IL-12 by cDC subsets *ex vivo* was in accordance with previous work [108], with the data here supplementing these observations by describing the inhibition of IL-12p70 production as a result of autocrine IL-10 signalling in cDCs during infection. In addition, infection led to substantial production of IL-27 by cDC subsets, providing the first evidence that experimental infection with *L. donovani* results in expression of this pleiotropic effector/regulatory cytokine at the protein level. The combination of enhanced IL-10 and IL-27 production, alongside suppressed IL-12 expression, inhibited the capacity for cDCs to drive Th1 polarisation *in vitro*, with further work suggesting an added function for IL-27 in directly limiting effector T cell polarisation (**6.1**).

It is still unclear as to how IL-27 can have such divergent effects on immune function, but with recent studies reporting that IL-27 can directly antagonise cytokine signalling [663] and that optimal IL-10 production by macrophages requires IL-27 expression [664], it is evident that this cytokine plays a central role in multiple immunoregulatory pathways. As the mechanisms underlying the inhibitory role of cDC-derived IL-10 and IL-27 during infection are still unclear, further studies are needed to determine whether cDCs, T cells or both require IL-10 and/or IL-27 receptor expression in order to establish the regulatory cytokine network proposed. Conditional ablation of these receptors on specific cell types would enable this to be addressed

The dramatic improvement in splenic pathology and disease progression after the ablation of CD11c-expressing cells *in vivo* strongly implicated DCs as contributors to the establishment of chronic infection. However, this experimental approach resulted in the depletion of many other CD11c-expressing cell populations – a caveat not addressed by previous *in vivo* ablation studies. As such, a major role for cDCs in disease progression was confirmed by the isolation of $CD11c^{hi}MHCII^{hi}$ cells from infected mice and their subsequent adoptive transfer to infected animals depleted of endogenous DC populations. Interestingly, transfer of $CD11c^{int}$ cells also led to a significant restoration of many elements of pathology, suggesting that cells within this heterogeneous population contribute to chronic infection with *L. donovani*. However, cDCs had a unique capacity to drive the differentiation of IFNγ and IL-10 dual producing $CD4^{+}$ T cells *in vivo*. Therefore, these combined depletion and complementation approaches indicate for the first time that $CD11c^{hi}$ cDCs can be detrimental to host protection during chronic infection and provide the first evidence that IL-10 and IL-27 producing

APCs critically contribute to the generation of IL-10 and IFNγ co-producing T cells *in vivo* (**6.2**).

An outstanding question of relevance to this study is whether individual cDC subsets make distinct contributions to pathology and disease progression during infection with *L. donovani*. It is not currently possible to perform the conditional ablation of individual cDC subsets *in vivo*; with the exception of partially depleting $CD8\alpha^+$ cDCs by cytochrome C injection [111]. However, mice constitutively lacking this subset are available [106] and a novel strain based on a similar inducible DTR system to that used here should allow for the conditional ablation of a range of $CD11c^+$ DC populations in the near future [665]. Until then it is difficult to speculate as to whether an individual subset may play a more significant role during infection. However, the relatively subtle phenotypic differences between the subsets *ex vivo* identified in this study suggest that some functional redundancy may exist.

Solid data regarding the question of why and how splenic cDC function is so profoundly altered during infection remain elusive. As cDCs themselves are rarely infected, the stromal compartment is likely to play a major role; highlighted by the impact of infection on splenic stroma and the subsequent expansion of regulatory DCs [172]. Bone marrow stromal cells are also heavily parasitised, hinting at the potential for alterations in cDC developmental microenvironments to impact upon their functionality after differentiation. However this is all speculative at this stage.

Complementing the data discussed above, this study identified a novel function for IRF7 in regulating the response of splenic cDCs to activation with TLR2 agonists *in vivo*. Although seemingly disconnected at first glance, a more complete understanding of the regulation of splenic cDC function is important in the context of chronic parasitic infection, as well as a multitude of other cases. Although the molecular mechanisms remain to be determined, the data presented here strongly indicate that IRF7 regulates costimulatory molecule expression by cDCs. Taken alongside bioinformatic data indicating that the promoters of various negative regulators of TLR signalling contain putative IRF7 binding sites (Phillips *et al*, unpublished), it is conceivable that this function may be due to the direct interaction of IRF7 and components of signalling pathways downstream of TLRs. A more complete characterisation of the targets of IRF7 and molecular mechanisms involved will be required to confirm this.

Any research focussing on a neglected tropical disease has an ultimate aim to generate a greater understanding of the pathological mechanisms involved and thus contribute to the development of potential novel therapeutic interventions. Although not focussed on directly applied topics such as vaccine development or drug discovery, this study made several novel observations regarding chronic *Leishmania donovani* infection that may impact upon therapeutic development in the future.

The paradoxical capacity for splenic cDCs to inhibit effective immune responses to *L. donovani* suggests that strategies aimed at harnessing the usually powerful stimulatory capacity of these cells would be ineffective if deployed against infection with this parasite. For example, delivery of antigen specifically to cDC subsets is currently in preclinical development for prophylactic vaccines against HIV, Malaria and pneumonic

plague [146, 666, 667]. Although these are not therapeutic vaccines, similar approaches administered during ongoing infection with *L. donovani* would be likely to fail as a result of the impaired cDC function identified in this study. Indeed, the rational design of therapeutic vaccine candidates that were specifically targeted to APCs other than DCs would appear to be the most sensible option, if pursued at all.

In light of the dramatic improvements in pathology and disease progression after ablation of cDCs during infection, a seemingly logical progression would be to consider the specific ablation of these cells during infection in humans. However, with no direct evidence of similarly impaired splenic DC function in patients as yet and the multitude of potential issues surrounding the depletion of crucial immune cell populations such as DCs, this is unlikely to be pursued. Nevertheless, the specific targeting of cDCs with pharmacological compounds, such as ERK inhibitors, is theoretically feasible: potentially allowing for the negative functions of these cells to be reversed. However in reality any strategies associated with the modulation of DC function are unlikely to be developed solely for diseases such as visceral leishmaniasis - a novel therapeutic would be required to have a primary indication for conditions associated with wealth, not poverty.

The evidence of a profound suppressive role for IL-10 at the level of the APC during *L. donovani* infection adds to the growing body of evidence supporting an assessment of biological anti-IL-10 therapy as an adjunct to chemotherapy in humans. Although this concept is strongly backed up by data from preclinical studies [321], any biological therapy is likely to have a limited impact on the burden of disease in endemic areas, again due to the extreme cost associated developing these products. However,

investigating DC function in groups of patients before and after anti-IL-10 therapy could address whether similar IL-10-mediated modulation of DC cytokine production is also a feature of human disease.

In summary, the data presented here reveal new insight into the immunopathological mechanisms underlying chronic infection with *Leishmania donovani*. The detrimental impact of cDCs on protective immunity suggests a previously unknown capacity for $CD11c^{hi}$ lymphoid resident DCs to regulate immune responses, indicating the considerable functional plasticity of these cells during infection. Although many outstanding questions remain, this study serves to highlight the diverse function of these fascinating cells across a spectrum of immune mediated disease and furthers our understanding of the immunoregulatory networks fundamental to our survival.

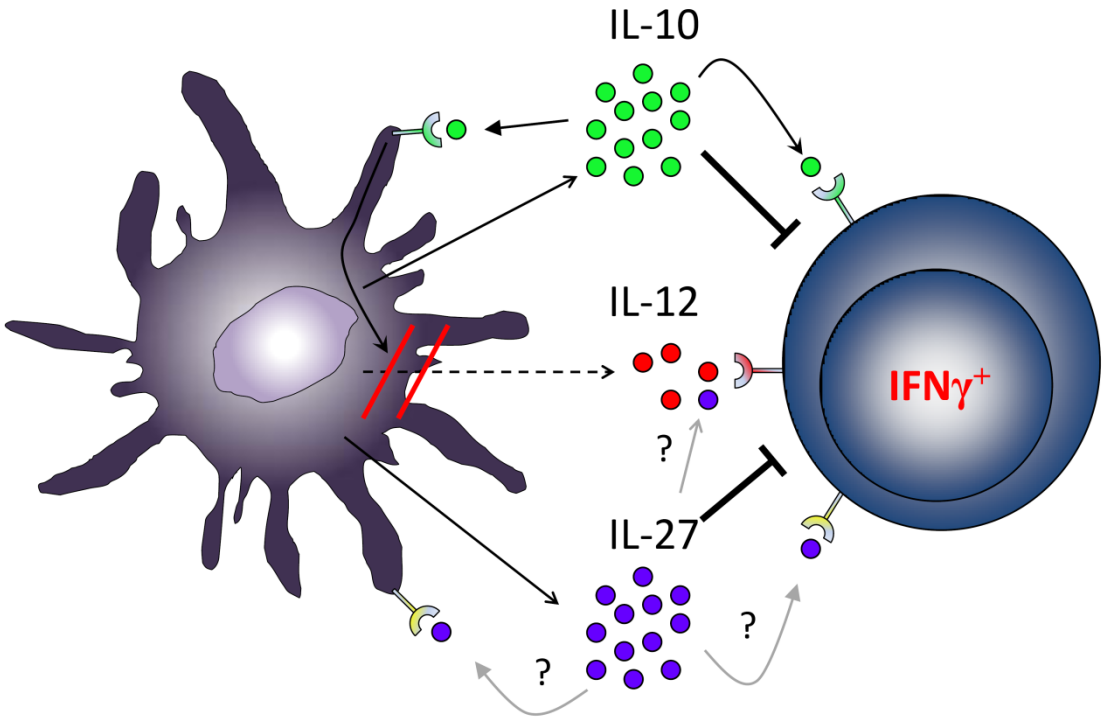

Figure 6.1 An autoregulatory cytokine cascade at the level of the cDC impairs efficient Th1 polarisation. Production of IL-10 by cDCs during chronic infection contributes to the inhibition of effector T cell polarisation by impairing IL-12 expression as a result of autocrine uptake by cDCs. It is feasible that cDC-derived IL-10 may additionally act directly on the T cell. IL-27 is also produced by cDCs as a result of infection, which contributes to the impaired Th1 polarisation by limiting IL-12 induced IFNγ production *in vitro* through an unknown mechanism, at least partially dependent upon cDC-derived IL-10.

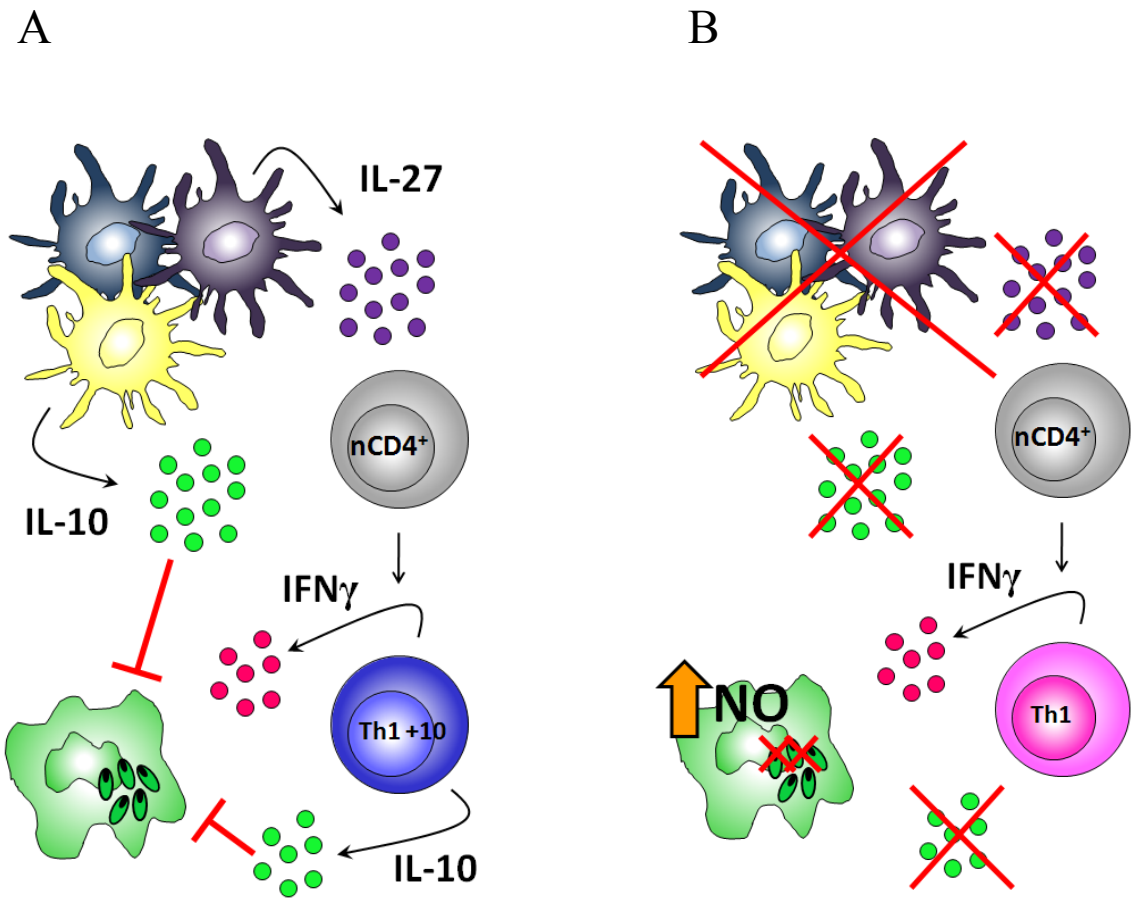

Figure 6.2 Model of splenic cDC function during chronic infection. A. cDC subsets produce IL-10 and IL-27 as a result of chronic infection, favouring the generation, maintenance and/or expansion of $CD4^+$ T cells capable of co-producing IFNγ and IL-10. Production of IL-10 by cDCs and $CD4^+$ T cells (also from other sources not depicted) impairs IFNγ-mediated parasite killing by macrophages, allowing for persistent disease. **B.** Conditional ablation of cDCs during infection removes a source of IL-10 and IL-27, limiting the numbers of IL-10^+ Th1 cells and reducing the overall abundance of IL-10. Alongside dramatic improvements in splenic pathology, residual levels of IFNγ allow for increased NO production by macrophages, parasite killing and enhanced host resistance.

Abbreviations

APC	Antigen presenting cell
APC	Allophycocyanin (fluorochrome)
B7-H1	see PD-L1
BM	Bone marrow
BMDC	Bone marrow derived dendritic cell
BrdU	5-bromo 2-deoxyuridine
BSA	Bovine serum albumin
CCR	Chemokine receptor
CD	Cluster of differentiation
cDC	Conventional $CD11c^{hi}MHCII^{hi}$ dendritic cell
CDP	Common dendritic cell progenitor
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DMEM	Dulbecco's modified eagle's medium
DN	Double negative
DNA	Deoxyribonucleic acid
DTR	Diphtheria toxin receptor
DTx	Diphtheria toxin
EAE	Experimental autoimmune encephalomyelitis
EBI3	Epstein-Barr virus-induced gene 3

ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal-related kinase
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Flt3	Fms-like tyrosine kinase receptor-3
Flt3L	Fms-like tyrosine kinase receptor-3 ligand
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK3	Glycogen synthase kinase-3
GVHD	Graft-versus host disease
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HTLV-1	Human T-lymphotropic virus
i.p	Intraperitoneal
i.v	Intravenous
ICOS	Inducible co-stimulatory molecule
iDC	Inflammatory monocyte-derived dendritic cell
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IgG	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISRE	Interferon stimulated response element
iTr35	Induced IL-35^{+} regulatory T cell
LC	Langerhans cell

LC	Langerhans cell
LCMV	Lymphocytic choriomeningitis virus
LDU	Leishman-Donovan Unit
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LTα	Lymphotoxin- α
MCMV	Murine cytomegalovirus
mDC	Myeloid dendritic cell
MDP	Macrophage-dendritic cell progenitor
MHC	Major Histocompatibility complex
MLR	Mixed leukocyte reaction
MMM	Marginal metallophillic macrophage
MMR	Macrophage mannose receptor
MONO	Monocyte
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
MZM	Marginal zone macrophage
NF-κB	Nuclear factor κB
NK	Natural killer
NO	Nitric oxide
OVA	Ovalbumin
p.s.	Post-stimulation
PAMP	Pathogen-associate molecular pattern
PBS	Phosphate-buffered saline
pDC	Plasmacytoid dendritic cell

PD-L1	Programmed death ligand 1
PE	Phycoerythrin
Pre-cDC	Conventional dendritic cell precursor
Pre-DC	Dendritic cell precursor
Pro-DC	Common dendritic cell progenitor (alternative nomenclature)
PRR	Pattern recognition receptor
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
RALDH	Retinaldehyde dehydrogenase
RORγt	Retinoic acid-related orphan receptor-γt
Sb^v	Sodium stibogluconate
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
TCR	T cell receptor
TGF	Transforming growth factor
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
Tip-DC	TNFα and iNOS-expressing inflammatory dendritic cell
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tr1	Regulatory T cell type 1
TRAF6	Tumour necrosis factor receptor-associated factor-6
Treg	Regulatory T cell
TRIM	Tripartite motif-containing protein

VEGF	Vascular endothelial growth factor
VL	Visceral leishmaniasis
WSX-1	IL-27 receptor α chain
WT	Wildtype
αIL-10R	anti-IL-10 receptor

References

1. Steinman, R.M. and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution.* J Exp Med, 1973. **137**(5): p. 1142-62.
2. Steinman, R.M. and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro.* J Exp Med, 1974. **139**(2): p. 380-97.
3. Steinman, R.M., D.S. Lustig, and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice. 3. Functional properties in vivo.* J Exp Med, 1974. **139**(6): p. 1431-45.
4. Steinman, R.M., J.C. Adams, and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice. IV. Identification and distribution in mouse spleen.* J Exp Med, 1975. **141**(4): p. 804-20.
5. Steinman, R.M. and M.D. Witmer, *Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice.* Proc Natl Acad Sci U S A, 1978. **75**(10): p. 5132-6.
6. Nussenzweig, M.C. and R.M. Steinman, *Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction.* J Exp Med, 1980. **151**(5): p. 1196-212.
7. Nussenzweig, M.C., et al., *Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes.* J Exp Med, 1980. **152**(4): p. 1070-84.
8. Metlay, J.P., et al., *The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies.* J Exp Med, 1990. **171**(5): p. 1753-71.
9. Steinman, R.M., *The dendritic cell system and its role in immunogenicity.* Annu Rev Immunol, 1991. **9**: p. 271-96.
10. Billingham, R.E., *Dendritic cells.* J Anat, 1948. **82**(Pt 1-2): p. 93-109.
11. Witmer-Pack, M.D., et al., *Quantitation of surface antigens on cultured murine epidermal Langerhans cells: rapid and selective increase in the level of surface MHC products.* J Invest Dermatol, 1988. **90**(3): p. 387-94.
12. Schuler, G. and R.M. Steinman, *Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro.* J Exp Med, 1985. **161**(3): p. 526-46.
13. Romani, N., et al., *Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells.* J Exp Med, 1989. **169**(3): p. 1169-78.
14. Inaba, K., et al., *The tissue distribution of the B7-2 costimulator in mice: abundant expression on dendritic cells in situ and during maturation in vitro.* J Exp Med, 1994. **180**(5): p. 1849-60.
15. Shimada, S., et al., *Enhanced antigen-presenting capacity of cultured Langerhans' cells is associated with markedly increased expression of Ia antigen.* J Immunol, 1987. **139**(8): p. 2551-5.
16. Pure, E., et al., *Antigen processing by epidermal Langerhans cells correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain.* J Exp Med, 1990. **172**(5): p. 1459-69.
17. Macatonia, S.E., et al., *Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate. Functional and morphological studies.* J Exp Med, 1987. **166**(6): p. 1654-67.
18. Guermonprez, P., et al., *Antigen presentation and T cell stimulation by dendritic cells.* Annual review of immunology, 2002. **20**: p. 621-67.
19. Pierre, P., et al., *Developmental regulation of MHC class II transport in mouse dendritic cells.* Nature, 1997. **388**(6644): p. 787-92.
20. Cella, M., et al., *Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells.* Nature, 1997. **388**(6644): p. 782-7.
21. Sharpe, A.H. and G.J. Freeman, *The B7-CD28 superfamily.* Nat Rev Immunol, 2002. **2**(2): p. 116-26.

22. Bretscher, P.A., *A two-step, two-signal model for the primary activation of precursor helper T cells.* Proc Natl Acad Sci U S A, 1999. **96**(1): p. 185-90.
23. Itano, A.A. and M.K. Jenkins, *Antigen presentation to naive CD4 T cells in the lymph node.* Nat Immunol, 2003. **4**(8): p. 733-9.
24. Dieu, M.C., et al., *Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites.* J Exp Med, 1998. **188**(2): p. 373-86.
25. Sozzani, S., et al., *Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties.* J Immunol, 1998. **161**(3): p. 1083-6.
26. Sallusto, F., et al., *Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation.* Eur J Immunol, 1998. **28**(9): p. 2760-9.
27. Gunn, M.D., et al., *Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization.* J Exp Med, 1999. **189**(3): p. 451-60.
28. Saeki, H., et al., *Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes.* J Immunol, 1999. **162**(5): p. 2472-5.
29. Wilson, N.S. and J.A. Villadangos, *Lymphoid organ dendritic cells: beyond the Langerhans cells paradigm.* Immunol Cell Biol, 2004. **82**(1): p. 91-8.
30. Shortman, K. and Y.J. Liu, *Mouse and human dendritic cell subtypes.* Nat Rev Immunol, 2002. **2**(3): p. 151-61.
31. Vremec, D., et al., *CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen.* J Immunol, 2000. **164**(6): p. 2978-86.
32. Winkel, K., et al., *CD4 and CD8 expression by human and mouse thymic dendritic cells.* Immunol Lett, 1994. **40**(2): p. 93-9.
33. Kondo, M., I.L. Weissman, and K. Akashi, *Identification of clonogenic common lymphoid progenitors in mouse bone marrow.* Cell, 1997. **91**(5): p. 661-72.
34. Akashi, K., et al., *A clonogenic common myeloid progenitor that gives rise to all myeloid lineages.* Nature, 2000. **404**(6774): p. 193-7.
35. Bell, J.J. and A. Bhandoola, *The earliest thymic progenitors for T cells possess myeloid lineage potential.* Nature, 2008. **452**(7188): p. 764-7.
36. Wada, H., et al., *Adult T-cell progenitors retain myeloid potential.* Nature, 2008. **452**(7188): p. 768-72.
37. Doulatov, S., et al., *Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development.* Nat Immunol, 2010. **11**(7): p. 585-93.
38. Wu, L., et al., *RelB is essential for the development of myeloid-related CD8alpha- dendritic cells but not of lymphoid-related CD8alpha+ dendritic cells.* Immunity, 1998. **9**(6): p. 839-47.
39. Guerriero, A., et al., *PU.1 is required for myeloid-derived but not lymphoid-derived dendritic cells.* Blood, 2000. **95**(3): p. 879-85.
40. Traver, D., et al., *Development of CD8alpha-positive dendritic cells from a common myeloid progenitor.* Science, 2000. **290**(5499): p. 2152-4.
41. Manz, M.G., et al., *Dendritic cell development from common myeloid progenitors.* Ann N Y Acad Sci, 2001. **938**: p. 167-73; discussion 173-4.
42. Manz, M.G., et al., *Dendritic cell potentials of early lymphoid and myeloid progenitors.* Blood, 2001. **97**(11): p. 3333-41.
43. Fogg, D.K., et al., *A clonogenic bone marrow progenitor specific for macrophages and dendritic cells.* Science, 2006. **311**(5757): p. 83-7.
44. Onai, N., et al., *Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow.* Nat Immunol, 2007. **8**(11): p. 1207-16.
45. Naik, S.H., et al., *Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo.* Nat Immunol, 2007. **8**(11): p. 1217-26.

46. del Hoyo, G.M., et al., *Characterization of a common precursor population for dendritic cells.* Nature, 2002. **415**(6875): p. 1043-7.
47. Naik, S.H., et al., *Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes.* Nat Immunol, 2006. **7**(6): p. 663-71.
48. Naik, S., et al., *CD8alpha+ mouse spleen dendritic cells do not originate from the CD8alpha- dendritic cell subset.* Blood, 2003. **102**(2): p. 601-4.
49. Liu, K., et al., *In vivo analysis of dendritic cell development and homeostasis.* Science, 2009. **324**(5925): p. 392-7.
50. McKenna, H.J., et al., *Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells.* Blood, 2000. **95**(11): p. 3489-97.
51. Karsunky, H., et al., *Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells in vivo.* J Exp Med, 2003. **198**(2): p. 305-13.
52. Onai, N., et al., *Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development.* J Exp Med, 2006. **203**(1): p. 227-38.
53. Waskow, C., et al., *The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues.* Nat Immunol, 2008. **9**(6): p. 676-83.
54. Kabashima, K., et al., *Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells.* Immunity, 2005. **22**(4): p. 439-50.
55. Kamath, A.T., et al., *Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs.* Blood, 2002. **100**(5): p. 1734-41.
56. Kamath, A.T., et al., *The development, maturation, and turnover rate of mouse spleen dendritic cell populations.* J Immunol, 2000. **165**(12): p. 6762-70.
57. Liu, K., et al., *Origin of dendritic cells in peripheral lymphoid organs of mice.* Nat Immunol, 2007. **8**(6): p. 578-83.
58. Kim, J.M., J.P. Rasmussen, and A.Y. Rudensky, *Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice.* Nat Immunol, 2007. **8**(2): p. 191-7.
59. Schildknecht, A., et al., *FoxP3+ regulatory T cells essentially contribute to peripheral CD8+ T-cell tolerance induced by steady-state dendritic cells.* Proc Natl Acad Sci U S A, 2010. **107**(1): p. 199-203.
60. Serbina, N.V. and E.G. Pamer, *Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2.* Nat Immunol, 2006. **7**(3): p. 311-7.
61. Geissmann, F., S. Jung, and D.R. Littman, *Blood monocytes consist of two principal subsets with distinct migratory properties.* Immunity, 2003. **19**(1): p. 71-82.
62. Randolph, G.J., et al., *Differentiation of phagocytic monocytes into lymph node dendritic cells in vivo.* Immunity, 1999. **11**(6): p. 753-61.
63. Cheong, C., et al., *Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209(+) dendritic cells for immune T cell areas.* Cell, 2010. **143**(3): p. 416-29.
64. Leon, B., M. Lopez-Bravo, and C. Ardavin, *Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania.* Immunity, 2007. **26**(4): p. 519-31.
65. Auffray, C., et al., *CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation.* J Exp Med, 2009. **206**(3): p. 595-606.
66. De Trez, C., et al., *iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic Leishmania major infection phase of C57BL/6 resistant mice.* PLoS Pathog, 2009. **5**(6): p. e1000494.
67. Bosschaerts, T., et al., *Tip-DC development during parasitic infection is regulated by IL-10 and requires CCL2/CCR2, IFN-gamma and MyD88 signaling.* PLoS Pathog, 2010. **6**(8).
68. Guilliams, M., et al., *IL-10 dampens TNF/inducible nitric oxide synthase-producing dendritic cell-mediated pathogenicity during parasitic infection.* J Immunol, 2009. **182**(2): p. 1107-18.

69. Copin, R., et al., *MyD88-dependent activation of B220-CD11b+LY-6C+ dendritic cells during Brucella melitensis infection.* J Immunol, 2007. **178**(8): p. 5182-91.
70. Jakubzick, C., et al., *Lymph-migrating, tissue-derived dendritic cells are minor constituents within steady-state lymph nodes.* J Exp Med, 2008. **205**(12): p. 2839-50.
71. Inaba, K., et al., *Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor.* J Exp Med, 1992. **176**(6): p. 1693-702.
72. Sallusto, F. and A. Lanzavecchia, *Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha.* J Exp Med, 1994. **179**(4): p. 1109-18.
73. Laouar, Y., et al., *STAT3 is required for Flt3L-dependent dendritic cell differentiation.* Immunity, 2003. **19**(6): p. 903-12.
74. Naik, S.H., et al., *Cutting edge: generation of splenic CD8+ and CD8- dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures.* J Immunol, 2005. **174**(11): p. 6592-7.
75. Brasel, K., et al., *Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures.* Blood, 2000. **96**(9): p. 3029-39.
76. Naik, S.H., et al., *CD8+, CD8-, and plasmacytoid dendritic cell generation in vitro using flt3 ligand.* Methods Mol Biol, 2010. **595**: p. 167-76.
77. Wong, P. and E.G. Pamer, *CD8 T cell responses to infectious pathogens.* Annual review of immunology, 2003. **21**: p. 29-70.
78. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* Journal of immunology, 1986. **136**(7): p. 2348-57.
79. Aggarwal, S., et al., *Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17.* The Journal of biological chemistry, 2003. **278**(3): p. 1910-4.
80. Veldhoen, M., et al., *Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset.* Nature immunology, 2008. **9**(12): p. 1341-6.
81. King, C., S.G. Tangye, and C.R. Mackay, *T follicular helper (TFH) cells in normal and dysregulated immune responses.* Annual review of immunology, 2008. **26**: p. 741-66.
82. Pulendran, B., et al., *Distinct dendritic cell subsets differentially regulate the class of immune response in vivo.* Proc Natl Acad Sci U S A, 1999. **96**(3): p. 1036-41.
83. Maldonado-Lopez, R., et al., *Role of CD8alpha+ and CD8alpha- dendritic cells in the induction of primary immune responses in vivo.* J Leukoc Biol, 1999. **66**(2): p. 242-6.
84. Maldonado-Lopez, R., et al., *CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo.* J Exp Med, 1999. **189**(3): p. 587-92.
85. De Smedt, T., et al., *CD8alpha(-) and CD8alpha(+) subclasses of dendritic cells undergo phenotypic and functional maturation in vitro and in vivo.* J Leukoc Biol, 2001. **69**(6): p. 951-8.
86. Dalod, M., et al., *Dendritic cell responses to early murine cytomegalovirus infection: subset functional specialization and differential regulation by interferon alpha/beta.* J Exp Med, 2003. **197**(7): p. 885-98.
87. Yrlid, U. and M.J. Wick, *Antigen presentation capacity and cytokine production by murine splenic dendritic cell subsets upon Salmonella encounter.* J Immunol, 2002. **169**(1): p. 108-16.
88. Reis e Sousa, C., et al., *In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas.* J Exp Med, 1997. **186**(11): p. 1819-29.
89. Edwards, A.D., et al., *Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines.* Eur J Immunol, 2003. **33**(4): p. 827-33.
90. Fujii, S., et al., *Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for*

combined CD4 and CD8 T cell immunity to a coadministered protein. J Exp Med, 2003. **198**(2): p. 267-79.
91. Maldonado-Lopez, R., et al., *Cytokines regulate the capacity of CD8alpha(+) and CD8alpha(-) dendritic cells to prime Th1/Th2 cells in vivo.* J Immunol, 2001. **167**(8): p. 4345-50.
92. Hochrein, H., et al., *Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets.* J Immunol, 2001. **166**(9): p. 5448-55.
93. Dudziak, D., et al., *Differential antigen processing by dendritic cell subsets in vivo.* Science, 2007. **315**(5808): p. 107-11.
94. den Haan, J.M., S.M. Lehar, and M.J. Bevan, *CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo.* J Exp Med, 2000. **192**(12): p. 1685-96.
95. Schnorrer, P., et al., *The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture.* Proc Natl Acad Sci U S A, 2006. **103**(28): p. 10729-34.
96. Sancho, D., et al., *Identification of a dendritic cell receptor that couples sensing of necrosis to immunity.* Nature, 2009. **458**(7240): p. 899-903.
97. Burgdorf, S., et al., *Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation.* Science, 2007. **316**(5824): p. 612-6.
98. Pooley, J.L., W.R. Heath, and K. Shortman, *Cutting edge: intravenous soluble antigen is presented to CD4 T cells by CD8- dendritic cells, but cross-presented to CD8 T cells by CD8+ dendritic cells.* J Immunol, 2001. **166**(9): p. 5327-30.
99. Allan, R.S., et al., *Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells.* Science, 2003. **301**(5641): p. 1925-8.
100. Allan, R.S., et al., *Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming.* Immunity, 2006. **25**(1): p. 153-62.
101. Belz, G.T., et al., *Cutting edge: conventional CD8 alpha+ dendritic cells are generally involved in priming CTL immunity to viruses.* J Immunol, 2004. **172**(4): p. 1996-2000.
102. Smith, C.M., et al., *Cutting edge: conventional CD8 alpha+ dendritic cells are preferentially involved in CTL priming after footpad infection with herpes simplex virus-1.* J Immunol, 2003. **170**(9): p. 4437-40.
103. Belz, G.T., et al., *CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo.* J Immunol, 2005. **175**(1): p. 196-200.
104. Yarovinsky, F., et al., *TLR11 activation of dendritic cells by a protozoan profilin-like protein.* Science, 2005. **308**(5728): p. 1626-9.
105. Gao, X., et al., *CD8+ DC, but Not CD8(-)DC, isolated from BCG-infected mice reduces pathological reactions induced by mycobacterial challenge infection.* PLoS One, 2010. **5**(2): p. e9281.
106. Hildner, K., et al., *Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity.* Science, 2008. **322**(5904): p. 1097-100.
107. Yarovinsky, F., et al., *Toll-like receptor recognition regulates immunodominance in an antimicrobial CD4+ T cell response.* Immunity, 2006. **25**(4): p. 655-64.
108. Maroof, A. and P.M. Kaye, *Temporal regulation of interleukin-12p70 (IL-12p70) and IL-12-related cytokines in splenic dendritic cell subsets during Leishmania donovani infection.* Infect Immun, 2008. **76**(1): p. 239-49.
109. den Haan, J.M. and M.J. Bevan, *Constitutive versus activation-dependent cross-presentation of immune complexes by CD8(+) and CD8(-) dendritic cells in vivo.* J Exp Med, 2002. **196**(6): p. 817-27.
110. Kamphorst, A.O., et al., *Route of antigen uptake differentially impacts presentation by dendritic cells and activated monocytes.* J Immunol, 2010. **185**(6): p. 3426-35.
111. Lin, M.L., et al., *Selective suicide of cross-presenting CD8+ dendritic cells by cytochrome c injection shows functional heterogeneity within this subset.* Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3029-34.
112. Sponaas, A.M., et al., *Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells.* J Exp Med, 2006. **203**(6): p. 1427-33.
113. Mount, A.M., et al., *Multiple dendritic cell populations activate CD4+ T cells after viral stimulation.* PLoS One, 2008. **3**(2): p. e1691.

114. Carter, R.W., et al., *Preferential induction of CD4+ T cell responses through in vivo targeting of antigen to dendritic cell-associated C-type lectin-1.* J Immunol, 2006. **177**(4): p. 2276-84.
115. Finkelman, F.D., et al., *Dendritic cells can present antigen in vivo in a tolerogenic or immunogenic fashion.* J Immunol, 1996. **157**(4): p. 1406-14.
116. Hawiger, D., et al., *Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo.* J Exp Med, 2001. **194**(6): p. 769-79.
117. Bonifaz, L., et al., *Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance.* J Exp Med, 2002. **196**(12): p. 1627-38.
118. Liu, K., et al., *Immune tolerance after delivery of dying cells to dendritic cells in situ.* J Exp Med, 2002. **196**(8): p. 1091-7.
119. Probst, H.C., et al., *Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4.* Nat Immunol, 2005. **6**(3): p. 280-6.
120. Wilson, N.S., D. El-Sukkari, and J.A. Villadangos, *Dendritic cells constitutively present self antigens in their immature state in vivo and regulate antigen presentation by controlling the rates of MHC class II synthesis and endocytosis.* Blood, 2004. **103**(6): p. 2187-95.
121. Volkmann, A., T. Zal, and B. Stockinger, *Antigen-presenting cells in the thymus that can negatively select MHC class II-restricted T cells recognizing a circulating self antigen.* J Immunol, 1997. **158**(2): p. 693-706.
122. Brocker, T., M. Riedinger, and K. Karjalainen, *Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo.* J Exp Med, 1997. **185**(3): p. 541-50.
123. Zal, T., A. Volkmann, and B. Stockinger, *Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen.* J Exp Med, 1994. **180**(6): p. 2089-99.
124. Steinman, R.M., D. Hawiger, and M.C. Nussenzweig, *Tolerogenic dendritic cells.* Annu Rev Immunol, 2003. **21**: p. 685-711.
125. Steinman, R.M., et al., *Dendritic cell function in vivo during the steady state: a role in peripheral tolerance.* Ann N Y Acad Sci, 2003. **987**: p. 15-25.
126. Steinman, R.M. and M.C. Nussenzweig, *Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance.* Proc Natl Acad Sci U S A, 2002. **99**(1): p. 351-8.
127. Mayerova, D., et al., *Langerhans cells activate naive self-antigen-specific CD8 T cells in the steady state.* Immunity, 2004. **21**(3): p. 391-400.
128. Kleindienst, P., et al., *Simultaneous induction of CD4 T cell tolerance and CD8 T cell immunity by semimature dendritic cells.* J Immunol, 2005. **174**(7): p. 3941-7.
129. Perez, V.L., et al., *Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement.* Immunity, 1997. **6**(4): p. 411-7.
130. Albert, M.L., M. Jegathesan, and R.B. Darnell, *Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells.* Nat Immunol, 2001. **2**(11): p. 1010-7.
131. Baban, B., et al., *A minor population of splenic dendritic cells expressing CD19 mediates IDO-dependent T cell suppression via type I IFN signaling following B7 ligation.* Int Immunol, 2005. **17**(7): p. 909-19.
132. Mellor, A.L., et al., *Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3 dioxygenase.* Int Immunol, 2004. **16**(10): p. 1391-401.
133. Mellor, A.L., et al., *Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion.* J Immunol, 2003. **171**(4): p. 1652-5.
134. Belz, G.T., et al., *The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens.* J Exp Med, 2002. **196**(8): p. 1099-104.

135. Qiu, C.H., et al., *Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens.* J Immunol, 2009. **182**(7): p. 4127-36.
136. Jiang, W., et al., *The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing.* Nature, 1995. **375**(6527): p. 151-5.
137. Kato, M., et al., *cDNA cloning of human DEC-205, a putative antigen-uptake receptor on dendritic cells.* Immunogenetics, 1998. **47**(6): p. 442-50.
138. Schulz, O. and C. Reis e Sousa, *Cross-presentation of cell-associated antigens by CD8alpha+ dendritic cells is attributable to their ability to internalize dead cells.* Immunology, 2002. **107**(2): p. 183-9.
139. Iyoda, T., et al., *The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo.* J Exp Med, 2002. **195**(10): p. 1289-302.
140. Valdez, Y., et al., *Major histocompatibility complex class II presentation of cell-associated antigen is mediated by CD8alpha+ dendritic cells in vivo.* J Exp Med, 2002. **195**(6): p. 683-94.
141. Mukhopadhaya, A., et al., *Selective delivery of beta cell antigen to dendritic cells in vivo leads to deletion and tolerance of autoreactive CD8+ T cells in NOD mice.* Proc Natl Acad Sci U S A, 2008. **105**(17): p. 6374-9.
142. Luckashenak, N., et al., *Constitutive crosspresentation of tissue antigens by dendritic cells controls CD8+ T cell tolerance in vivo.* Immunity, 2008. **28**(4): p. 521-32.
143. Bonifaz, L.C., et al., *In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination.* J Exp Med, 2004. **199**(6): p. 815-24.
144. Mahnke, K., et al., *Targeting of antigens to activated dendritic cells in vivo cures metastatic melanoma in mice.* Cancer Res, 2005. **65**(15): p. 7007-12.
145. Trumpfheller, C., et al., *Intensified and protective CD4+ T cell immunity in mice with anti-dendritic cell HIV gag fusion antibody vaccine.* J Exp Med, 2006. **203**(3): p. 607-17.
146. Cheong, C., et al., *Improved cellular and humoral immune responses in vivo following targeting of HIV Gag to dendritic cells within human anti-human DEC205 monoclonal antibody.* Blood, 2010. **116**(19): p. 3828-38.
147. Yamazaki, S., et al., *CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells.* J Immunol, 2008. **181**(10): p. 6923-33.
148. Yamazaki, S., et al., *Dendritic cells are specialized accessory cells along with TGF- for the differentiation of Foxp3+ CD4+ regulatory T cells from peripheral Foxp3 precursors.* Blood, 2007. **110**(13): p. 4293-302.
149. Dhodapkar, M.V. and R.M. Steinman, *Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans.* Blood, 2002. **100**(1): p. 174-7.
150. Dhodapkar, M.V., et al., *Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells.* J Exp Med, 2001. **193**(2): p. 233-8.
151. Jonuleit, H., et al., *Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells.* J Exp Med, 2000. **192**(9): p. 1213-22.
152. Kretschmer, K., et al., *Inducing and expanding regulatory T cell populations by foreign antigen.* Nat Immunol, 2005. **6**(12): p. 1219-27.
153. Knoechel, B., et al., *Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen.* J Exp Med, 2005. **202**(10): p. 1375-86.
154. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
155. Khattri, R., et al., *An essential role for Scurfin in CD4+CD25+ T regulatory cells.* Nat Immunol, 2003. **4**(4): p. 337-42.
156. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
157. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.

158. Groux, H., et al., *A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis.* Nature, 1997. **389**(6652): p. 737-42.
159. Vieira, P.L., et al., *IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells.* J Immunol, 2004. **172**(10): p. 5986-93.
160. Collison, L.W., et al., *IL-35-mediated induction of a potent regulatory T cell population.* Nat Immunol, 2010. **11**(12): p. 1093-101.
161. Anderson, C.F., et al., *CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis.* J Exp Med, 2007. **204**(2): p. 285-97.
162. Jankovic, D., et al., *Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection.* J Exp Med, 2007. **204**(2): p. 273-83.
163. Coombes, J.L., et al., *A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism.* J Exp Med, 2007. **204**(8): p. 1757-64.
164. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid.* J Exp Med, 2007. **204**(8): p. 1775-85.
165. Toubai, T., et al., *Immunization with host-type CD8{alpha}+ dendritic cells reduces experimental acute GVHD in an IL-10-dependent manner.* Blood, 2010. **115**(3): p. 724-35.
166. Legge, K.L., et al., *On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity.* J Exp Med, 2002. **196**(2): p. 217-27.
167. Wakkach, A., et al., *Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo.* Immunity, 2003. **18**(5): p. 605-17.
168. Delgado, M., E. Gonzalez-Rey, and D. Ganea, *The neuropeptide vasoactive intestinal peptide generates tolerogenic dendritic cells.* J Immunol, 2005. **175**(11): p. 7311-24.
169. Fujita, S., et al., *Regulatory dendritic cells act as regulators of acute lethal systemic inflammatory response.* Blood, 2006. **107**(9): p. 3656-64.
170. Wong, K.A. and A. Rodriguez, *Plasmodium infection and endotoxic shock induce the expansion of regulatory dendritic cells.* J Immunol, 2008. **180**(2): p. 716-26.
171. Ocana-Morgner, C., et al., *Role of TGF-beta and PGE2 in T cell responses during Plasmodium yoelii infection.* Eur J Immunol, 2007. **37**(6): p. 1562-74.
172. Svensson, M., et al., *Stromal cells direct local differentiation of regulatory dendritic cells.* Immunity, 2004. **21**(6): p. 805-16.
173. Nguyen Hoang, A.T., et al., *Stromal cell-derived CXCL12 and CCL8 cooperate to support increased development of regulatory dendritic cells following Leishmania infection.* J Immunol, 2010. **185**(4): p. 2360-71.
174. McGuirk, P., C. McCann, and K.H. Mills, *Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis.* J Exp Med, 2002. **195**(2): p. 221-31.
175. Higgins, S.C., et al., *Toll-like receptor 4-mediated innate IL-10 activates antigen-specific regulatory T cells and confers resistance to Bordetella pertussis by inhibiting inflammatory pathology.* J Immunol, 2003. **171**(6): p. 3119-27.
176. van der Kleij, D., et al., *A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization.* J Biol Chem, 2002. **277**(50): p. 48122-9.
177. Cuellar, C., W. Wu, and S. Mendez, *The hookworm tissue inhibitor of metalloproteases (Ac-TMP-1) modifies dendritic cell function and induces generation of CD4 and CD8 suppressor T cells.* PLoS Negl Trop Dis, 2009. **3**(5): p. e439.
178. Depaolo, R.W., et al., *Toll-like receptor 6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis.* Cell Host Microbe, 2008. **4**(4): p. 350-61.
179. Hashimoto, C., K.L. Hudson, and K.V. Anderson, *The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein.* Cell, 1988. **52**(2): p. 269-79.

180. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-84.
181. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
182. Schaefer, L., et al., *The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages.* J Clin Invest, 2005. **115**(8): p. 2223-33.
183. Jiang, D., et al., *Regulation of lung injury and repair by Toll-like receptors and hyaluronan.* Nat Med, 2005. **11**(11): p. 1173-9.
184. Vollmer, J., et al., *Immune stimulation mediated by autoantigen binding sites within small nuclear RNAs involves Toll-like receptors 7 and 8.* J Exp Med, 2005. **202**(11): p. 1575-85.
185. Means, T.K., et al., *Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9.* J Clin Invest, 2005. **115**(2): p. 407-17.
186. Kim, S., et al., *Carcinoma-produced factors activate myeloid cells through TLR2 to stimulate metastasis.* Nature, 2009. **457**(7225): p. 102-6.
187. Wesche, H., et al., *MyD88: an adapter that recruits IRAK to the IL-1 receptor complex.* Immunity, 1997. **7**(6): p. 837-47.
188. Yamamoto, M., et al., *Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4.* Nature, 2002. **420**(6913): p. 324-9.
189. Horng, T., G.M. Barton, and R. Medzhitov, *TIRAP: an adapter molecule in the Toll signaling pathway.* Nat Immunol, 2001. **2**(9): p. 835-41.
190. Li, S., et al., *IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase.* Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5567-72.
191. Deng, L., et al., *Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain.* Cell, 2000. **103**(2): p. 351-61.
192. Wang, C., et al., *TAK1 is a ubiquitin-dependent kinase of MKK and IKK.* Nature, 2001. **412**(6844): p. 346-51.
193. Yamamoto, M., et al., *Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta.* Nature, 2004. **430**(6996): p. 218-22.
194. Palsson-McDermott, E.M., et al., *TAG, a splice variant of the adaptor TRAM, negatively regulates the adaptor MyD88-independent TLR4 pathway.* Nat Immunol, 2009. **10**(6): p. 579-86.
195. Shi, M., et al., *TRIM30 alpha negatively regulates TLR-mediated NF-kappa B activation by targeting TAB2 and TAB3 for degradation.* Nat Immunol, 2008. **9**(4): p. 369-77.
196. Sheedy, F.J., et al., *Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21.* Nat Immunol, 2010. **11**(2): p. 141-7.
197. Matsushita, K., et al., *Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay.* Nature, 2009. **458**(7242): p. 1185-90.
198. Sing, A., et al., *Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression.* J Exp Med, 2002. **196**(8): p. 1017-24.
199. Diterich, I., et al., *Borrelia burgdorferi-induced tolerance as a model of persistence via immunosuppression.* Infect Immun, 2003. **71**(7): p. 3979-87.
200. Netea, M.G., et al., *Aspergillus fumigatus evades immune recognition during germination through loss of toll-like receptor-4-mediated signal transduction.* J Infect Dis, 2003. **188**(2): p. 320-6.
201. Netea, M.G., et al., *Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells.* J Immunol, 2004. **172**(6): p. 3712-8.
202. Jang, S., et al., *IL-6 and IL-10 induction from dendritic cells in response to Mycobacterium tuberculosis is predominantly dependent on TLR2-mediated recognition.* J Immunol, 2004. **173**(5): p. 3392-7.
203. Slack, E.C., et al., *Syk-dependent ERK activation regulates IL-2 and IL-10 production by DC stimulated with zymosan.* Eur J Immunol, 2007. **37**(6): p. 1600-12.

204. Dillon, S., et al., *A Toll-like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells.* J Immunol, 2004. **172**(8): p. 4733-43.
205. Brown, G.D. and S. Gordon, *Immune recognition. A new receptor for beta-glucans.* Nature, 2001. **413**(6851): p. 36-7.
206. Rogers, N.C., et al., *Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins.* Immunity, 2005. **22**(4): p. 507-17.
207. Brown, G.D., et al., *Dectin-1 mediates the biological effects of beta-glucans.* J Exp Med, 2003. **197**(9): p. 1119-24.
208. Dillon, S., et al., *Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance.* J Clin Invest, 2006. **116**(4): p. 916-28.
209. Manicassamy, S., et al., *Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity.* Nat Med, 2009. **15**(4): p. 401-9.
210. Kroening, P.R., et al., *Cigarette smoke-induced oxidative stress suppresses generation of dendritic cell IL-12 and IL-23 through ERK-dependent pathways.* J Immunol, 2008. **181**(2): p. 1536-47.
211. Correa, F., et al., *Activation of cannabinoid CB2 receptor negatively regulates IL-12p40 production in murine macrophages: role of IL-10 and ERK1/2 kinase signaling.* Br J Pharmacol, 2005. **145**(4): p. 441-8.
212. Yi, A.K., et al., *Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response.* J Immunol, 2002. **168**(9): p. 4711-20.
213. Sutterwala, F.S., et al., *Reversal of proinflammatory responses by ligating the macrophage Fcgamma receptor type I.* J Exp Med, 1998. **188**(1): p. 217-22.
214. Lucas, M., et al., *ERK activation following macrophage FcgammaR ligation leads to chromatin modifications at the IL-10 locus.* J Immunol, 2005. **175**(1): p. 469-77.
215. Bandukwala, H.S., et al., *Signaling through Fc gamma RIII is required for optimal T helper type (Th)2 responses and Th2-mediated airway inflammation.* J Exp Med, 2007. **204**(8): p. 1875-89.
216. Anderson, C.F., et al., *T cell biasing by activated dendritic cells.* J Immunol, 2004. **173**(2): p. 955-61.
217. Ohtani, M., et al., *Mammalian target of rapamycin and glycogen synthase kinase 3 differentially regulate lipopolysaccharide-induced interleukin-12 production in dendritic cells.* Blood, 2008. **112**(3): p. 635-43.
218. Pflanz, S., et al., *IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells.* Immunity, 2002. **16**(6): p. 779-90.
219. Sprecher, C.A., et al., *Cloning and characterization of a novel class I cytokine receptor.* Biochem Biophys Res Commun, 1998. **246**(1): p. 82-90.
220. Chen, Q., et al., *Development of Th1-type immune responses requires the type I cytokine receptor TCCR.* Nature, 2000. **407**(6806): p. 916-20.
221. Yoshida, H., et al., *WSX-1 is required for the initiation of Th1 responses and resistance to L. major infection.* Immunity, 2001. **15**(4): p. 569-78.
222. Pflanz, S., et al., *WSX-1 and glycoprotein 130 constitute a signal-transducing receptor for IL-27.* J Immunol, 2004. **172**(4): p. 2225-31.
223. Owaki, T., et al., *IL-27 induces Th1 differentiation via p38 MAPK/T-bet- and intercellular adhesion molecule-1/LFA-1/ERK1/2-dependent pathways.* J Immunol, 2006. **177**(11): p. 7579-87.
224. Kamiya, S., et al., *An indispensable role for STAT1 in IL-27-induced T-bet expression but not proliferation of naive CD4+ T cells.* J Immunol, 2004. **173**(6): p. 3871-7.
225. Takeda, A., et al., *Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment.* J Immunol, 2003. **170**(10): p. 4886-90.

226. Lucas, S., et al., *IL-27 regulates IL-12 responsiveness of naive CD4+ T cells through Stat1-dependent and -independent mechanisms.* Proc Natl Acad Sci U S A, 2003. **100**(25): p. 15047-52.
227. Hibbert, L., et al., *IL-27 and IFN-alpha signal via Stat1 and Stat3 and induce T-Bet and IL-12Rbeta2 in naive T cells.* J Interferon Cytokine Res, 2003. **23**(9): p. 513-22.
228. Cao, Y., et al., *IL-27 induces a Th1 immune response and susceptibility to experimental arthritis.* J Immunol, 2008. **180**(2): p. 922-30.
229. Shainheit, M.G., et al., *Disruption of interleukin-27 signaling results in impaired gamma interferon production but does not significantly affect immunopathology in murine schistosome infection.* Infect Immun, 2007. **75**(6): p. 3169-77.
230. Honda, K., et al., *T helper 1-inducing property of IL-27/WSX-1 signaling is required for the induction of experimental colitis.* Inflamm Bowel Dis, 2005. **11**(12): p. 1044-52.
231. Goldberg, R., et al., *Suppression of ongoing experimental autoimmune encephalomyelitis by neutralizing the function of the p28 subunit of IL-27.* J Immunol, 2004. **173**(10): p. 6465-71.
232. Bancroft, A.J., et al., *WSX-1: a key role in induction of chronic intestinal nematode infection.* J Immunol, 2004. **172**(12): p. 7635-41.
233. Larousserie, F., et al., *Expression of IL-27 in human Th1-associated granulomatous diseases.* J Pathol, 2004. **202**(2): p. 164-71.
234. Goldberg, R., et al., *Suppression of ongoing adjuvant-induced arthritis by neutralizing the function of the p28 subunit of IL-27.* J Immunol, 2004. **173**(2): p. 1171-8.
235. Siebler, J., et al., *Cutting edge: a key pathogenic role of IL-27 in T cell- mediated hepatitis.* J Immunol, 2008. **180**(1): p. 30-3.
236. Mayer, K.D., et al., *Cutting edge: T-bet and IL-27R are critical for in vivo IFN-gamma production by CD8 T cells during infection.* J Immunol, 2008. **180**(2): p. 693-7.
237. Wang, R., et al., *The pathogenic role of interleukin-27 in autoimmune diabetes.* Cell Mol Life Sci, 2008. **65**(23): p. 3851-60.
238. Villarino, A., et al., *The IL-27R (WSX-1) is required to suppress T cell hyperactivity during infection.* Immunity, 2003. **19**(5): p. 645-55.
239. Hamano, S., et al., *WSX-1 is required for resistance to Trypanosoma cruzi infection by regulation of proinflammatory cytokine production.* Immunity, 2003. **19**(5): p. 657-67.
240. Yoshimura, T., et al., *Two-sided roles of IL-27: induction of Th1 differentiation on naive CD4+ T cells versus suppression of proinflammatory cytokine production including IL-23-induced IL-17 on activated CD4+ T cells partially through STAT3-dependent mechanism.* J Immunol, 2006. **177**(8): p. 5377-85.
241. Villarino, A.V., et al., *IL-27 limits IL-2 production during Th1 differentiation.* J Immunol, 2006. **176**(1): p. 237-47.
242. Owaki, T., et al., *IL-27 suppresses CD28-mediated [correction of medicated] IL-2 production through suppressor of cytokine signaling 3.* J Immunol, 2006. **176**(5): p. 2773-80.
243. Niedbala, W., et al., *Interleukin 27 attenuates collagen-induced arthritis.* Ann Rheum Dis, 2008. **67**(10): p. 1474-9.
244. Batten, M., et al., *Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells.* Nat Immunol, 2006. **7**(9): p. 929-36.
245. Stumhofer, J.S., et al., *Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system.* Nat Immunol, 2006. **7**(9): p. 937-45.
246. Fitzgerald, D.C., et al., *Suppressive effect of IL-27 on encephalitogenic Th17 cells and the effector phase of experimental autoimmune encephalomyelitis.* J Immunol, 2007. **179**(5): p. 3268-75.
247. Neufert, C., et al., *IL-27 controls the development of inducible regulatory T cells and Th17 cells via differential effects on STAT1.* Eur J Immunol, 2007. **37**(7): p. 1809-16.
248. Kimura, A., T. Naka, and T. Kishimoto, *IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells.* Proc Natl Acad Sci U S A, 2007. **104**(29): p. 12099-104.

249. Diveu, C., et al., *IL-27 blocks RORc expression to inhibit lineage commitment of Th17 cells.* J Immunol, 2009. **182**(9): p. 5748-56.
250. Guo, B., E.Y. Chang, and G. Cheng, *The type I IFN induction pathway constrains Th17-mediated autoimmune inflammation in mice.* J Clin Invest, 2008. **118**(5): p. 1680-90.
251. Shinohara, M.L., et al., *Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin.* Immunity, 2008. **29**(1): p. 68-78.
252. Zhang, X., et al., *IFN-beta1a inhibits the secretion of Th17-polarizing cytokines in human dendritic cells via TLR7 up-regulation.* J Immunol, 2009. **182**(6): p. 3928-36.
253. El-behi, M., et al., *Differential effect of IL-27 on developing versus committed Th17 cells.* J Immunol, 2009. **183**(8): p. 4957-67.
254. Awasthi, A., et al., *A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells.* Nat Immunol, 2007. **8**(12): p. 1380-9.
255. Fitzgerald, D.C., et al., *Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells.* Nat Immunol, 2007. **8**(12): p. 1372-9.
256. Stumhofer, J.S., et al., *Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10.* Nat Immunol, 2007. **8**(12): p. 1363-71.
257. Murugaiyan, G., et al., *IL-27 Is a Key Regulator of IL-10 and IL-17 Production by Human CD4+ T Cells.* J Immunol, 2009.
258. Spolski, R., et al., *IL-21 mediates suppressive effects via its induction of IL-10.* J Immunol, 2009. **182**(5): p. 2859-67.
259. Apetoh, L., et al., *The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27.* Nat Immunol, 2010. **11**(9): p. 854-61.
260. Pot, C., et al., *Cutting edge: IL-27 induces the transcription factor c-Maf, cytokine IL-21, and the costimulatory receptor ICOS that coordinately act together to promote differentiation of IL-10-producing Tr1 cells.* J Immunol, 2009. **183**(2): p. 797-801.
261. Hedrich, C.M., et al., *Dynamic DNA methylation patterns across the mouse and human IL10 genes during CD4(+) T cell activation; influence of IL-27.* Mol Immunol, 2010.
262. Batten, M., et al., *Cutting edge: IL-27 is a potent inducer of IL-10 but not FoxP3 in murine T cells.* J Immunol, 2008. **180**(5): p. 2752-6.
263. Rutz, S., et al., *Notch regulates IL-10 production by T helper 1 cells.* Proc Natl Acad Sci U S A, 2008. **105**(9): p. 3497-502.
264. Ouaked, N., et al., *Regulation of the foxp3 gene by the Th1 cytokines: the role of IL-27-induced STAT1.* J Immunol, 2009. **182**(2): p. 1041-9.
265. Huber, M., et al., *IL-27 inhibits the development of regulatory T cells via STAT3.* Int Immunol, 2008. **20**(2): p. 223-34.
266. Xu, L., et al., *Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I.* Immunity, 2010. **33**(3): p. 313-25.
267. Ilarregui, J.M., et al., *Tolerogenic signals delivered by dendritic cells to T cells through a galectin-1-driven immunoregulatory circuit involving interleukin 27 and interleukin 10.* Nat Immunol, 2009. **10**(9): p. 981-91.
268. Shiokawa, A., et al., *IL-10 and IL-27 producing dendritic cells capable of enhancing IL-10 production of T cells are induced in oral tolerance.* Immunol Lett, 2009. **125**(1): p. 7-14.
269. Herwaldt, B.L., *Leishmaniasis.* Lancet, 1999. **354**(9185): p. 1191-9.
270. Pearson, R.D. and A.Q. Sousa, *Clinical spectrum of Leishmaniasis.* Clin Infect Dis, 1996. **22**(1): p. 1-13.
271. Murray, H.W., et al., *Advances in leishmaniasis.* Lancet, 2005. **366**(9496): p. 1561-77.
272. Chappuis, F., et al., *Visceral leishmaniasis: what are the needs for diagnosis, treatment and control?* Nat Rev Microbiol, 2007. **5**(11): p. 873-82.
273. Zijlstra, E.E. and A.M. el-Hassan, *Leishmaniasis in Sudan. Visceral leishmaniasis.* Trans R Soc Trop Med Hyg, 2001. **95 Suppl 1**: p. S27-58.

274. Bern, C., et al., *Risk factors for kala-azar in Bangladesh.* Emerg Infect Dis, 2005. **11**(5): p. 655-62.
275. Lukes, J., et al., *Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy.* Proc Natl Acad Sci U S A, 2007. **104**(22): p. 9375-80.
276. Desjeux, P., *Leishmaniasis: current situation and new perspectives.* Comp Immunol Microbiol Infect Dis, 2004. **27**(5): p. 305-18.
277. Collin, S.M., et al., *Unseen Kala-azar deaths in south Sudan (1999-2002).* Trop Med Int Health, 2006. **11**(4): p. 509-12.
278. Singh, V.P., et al., *Estimation of under-reporting of visceral leishmaniasis cases in Bihar, India.* Am J Trop Med Hyg, 2010. **82**(1): p. 9-11.
279. Singh, S.P., et al., *Serious underreporting of visceral leishmaniasis through passive case reporting in Bihar, India.* Trop Med Int Health, 2006. **11**(6): p. 899-905.
280. Hasker, E., et al., *Management of visceral leishmaniasis in rural primary health care services in Bihar, India.* Trop Med Int Health, 2010. **15 Suppl 2**: p. 55-62.
281. Meheus, F., et al., *Costs of patient management of visceral leishmaniasis in Muzaffarpur, Bihar, India.* Trop Med Int Health, 2006. **11**(11): p. 1715-24.
282. Sundar, S., et al., *Resistance to treatment in Kala-azar: speciation of isolates from northeast India.* Am J Trop Med Hyg, 2001. **65**(3): p. 193-6.
283. Sundar, S., et al., *Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic.* Clin Infect Dis, 2000. **31**(4): p. 1104-7.
284. Gasser, R.A., Jr., et al., *Pancreatitis induced by pentavalent antimonial agents during treatment of leishmaniasis.* Clin Infect Dis, 1994. **18**(1): p. 83-90.
285. Maheshwari, A., et al., *Cumulative Cardiac Toxicity of Sodium Stibogluconate and Amphotericin B in Treatment of Kala-Azar.* Pediatr Infect Dis J, 2010.
286. Thakur, C.P., et al., *Do the diminishing efficacy and increasing toxicity of sodium stibogluconate in the treatment of visceral leishmaniasis in Bihar, India, justify its continued use as a first-line drug? An observational study of 80 cases.* Ann Trop Med Parasitol, 1998. **92**(5): p. 561-9.
287. Chappuis, F., et al., *High mortality among older patients treated with pentavalent antimonials for visceral leishmaniasis in East Africa and rationale for switch to liposomal amphotericin B.* Antimicrob Agents Chemother, 2010.
288. Davidson, R.N., et al., *Liposomal amphotericin B in drug-resistant visceral leishmaniasis.* Lancet, 1991. **337**(8749): p. 1061-2.
289. Croft, S.L., R.N. Davidson, and E.A. Thornton, *Liposomal amphotericin B in the treatment of visceral leishmaniasis.* J Antimicrob Chemother, 1991. **28 Suppl B**: p. 111-8.
290. Sundar, S., et al., *Treatment of Indian visceral leishmaniasis with single or daily infusions of low dose liposomal amphotericin B: randomised trial.* BMJ, 2001. **323**(7310): p. 419-22.
291. Sundar, S., et al., *Single-dose liposomal amphotericin B for visceral leishmaniasis in India.* N Engl J Med, 2010. **362**(6): p. 504-12.
292. Jha, T.K., et al., *Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis.* N Engl J Med, 1999. **341**(24): p. 1795-800.
293. Jha, T.K., et al., *Randomised controlled trial of aminosidine (paromomycin) v sodium stibogluconate for treating visceral leishmaniasis in North Bihar, India.* BMJ, 1998. **316**(7139): p. 1200-5.
294. Sundar, S., et al., *Injectable paromomycin for Visceral leishmaniasis in India.* N Engl J Med, 2007. **356**(25): p. 2571-81.
295. Hailu, A., et al., *Geographical variation in the response of visceral leishmaniasis to paromomycin in East Africa: a multicentre, open-label, randomized trial.* PLoS Negl Trop Dis, 2010. **4**(10): p. e709.
296. van Griensven, J., et al., *Combination therapy for visceral leishmaniasis.* Lancet Infect Dis, 2010. **10**(3): p. 184-94.
297. Kaye, P.M., et al., *The immunopathology of experimental visceral leishmaniasis.* Immunol Rev, 2004. **201**: p. 239-53.

298. Ahmed, S., et al., *Intradermal infection model for pathogenesis and vaccine studies of murine visceral leishmaniasis.* Infect Immun, 2003. **71**(1): p. 401-10.
299. Engwerda, C.R. and P.M. Kaye, *Organ-specific immune responses associated with infectious disease.* Immunol Today, 2000. **21**(2): p. 73-8.
300. Murray, H.W., et al., *Visceral leishmaniasis in mice devoid of tumor necrosis factor and response to treatment.* Infect Immun, 2000. **68**(11): p. 6289-93.
301. Tumang, M.C., et al., *Role and effect of TNF-alpha in experimental visceral leishmaniasis.* J Immunol, 1994. **153**(2): p. 768-75.
302. Engwerda, C.R., et al., *Distinct roles for lymphotoxin-alpha and tumor necrosis factor in the control of Leishmania donovani infection.* Am J Pathol, 2004. **165**(6): p. 2123-33.
303. Murray, H.W. and C.F. Nathan, *Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral Leishmania donovani.* J Exp Med, 1999. **189**(4): p. 741-6.
304. Kaye, P.M. and G.J. Bancroft, *Leishmania donovani infection in scid mice: lack of tissue response and in vivo macrophage activation correlates with failure to trigger natural killer cell-derived gamma interferon production in vitro.* Infect Immun, 1992. **60**(10): p. 4335-42.
305. Stern, J.J., et al., *Role of L3T4+ and LyT-2+ cells in experimental visceral leishmaniasis.* J Immunol, 1988. **140**(11): p. 3971-7.
306. Murray, H.W., et al., *Experimental visceral leishmaniasis: production of interleukin 2 and interferon-gamma, tissue immune reaction, and response to treatment with interleukin 2 and interferon-gamma.* J Immunol, 1987. **138**(7): p. 2290-7.
307. Bonventre, P.F. and A.D. Nickol, *Leishmania donovani infection in athymic mice derived from parental strains of the susceptible (Lshs) or resistant (Lshr) phenotype.* J Leukoc Biol, 1984. **36**(5): p. 651-8.
308. Engwerda, C.R., M. Ato, and P.M. Kaye, *Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis.* Trends Parasitol, 2004. **20**(11): p. 524-30.
309. Cotterell, S.E., C.R. Engwerda, and P.M. Kaye, *Leishmania donovani infection of bone marrow stromal macrophages selectively enhances myelopoiesis, by a mechanism involving GM-CSF and TNF-alpha.* Blood, 2000. **95**(5): p. 1642-51.
310. Cotterell, S.E., C.R. Engwerda, and P.M. Kaye, *Enhanced hematopoietic activity accompanies parasite expansion in the spleen and bone marrow of mice infected with Leishmania donovani.* Infect Immun, 2000. **68**(4): p. 1840-8.
311. Engwerda, C.R., et al., *Neutralization of IL-12 demonstrates the existence of discrete organ-specific phases in the control of Leishmania donovani.* Eur J Immunol, 1998. **28**(2): p. 669-80.
312. Smelt, S.C., et al., *Destruction of follicular dendritic cells during chronic visceral leishmaniasis.* J Immunol, 1997. **158**(8): p. 3813-21.
313. Ato, M., et al., *Defective CCR7 expression on dendritic cells contributes to the development of visceral leishmaniasis.* Nat Immunol, 2002. **3**(12): p. 1185-91.
314. Engwerda, C.R., et al., *A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during Leishmania donovani infection.* Am J Pathol, 2002. **161**(2): p. 429-37.
315. Smelt, S.C., et al., *B cell-deficient mice are highly resistant to Leishmania donovani infection, but develop neutrophil-mediated tissue pathology.* J Immunol, 2000. **164**(7): p. 3681-8.
316. Deak, E., et al., *Murine visceral leishmaniasis: IgM and polyclonal B-cell activation lead to disease exacerbation.* Eur J Immunol, 2010. **40**(5): p. 1355-68.
317. Blackwell, J.M., *Genetic susceptibility to leishmanial infections: studies in mice and man.* Parasitology, 1996. **112 Suppl**: p. S67-74.
318. Kaye, P.M., A.J. Curry, and J.M. Blackwell, *Differential production of Th1- and Th2-derived cytokines does not determine the genetically controlled or vaccine-induced rate of cure in murine visceral leishmaniasis.* J Immunol, 1991. **146**(8): p. 2763-70.
319. Miralles, G.D., et al., *Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis.* Infect Immun, 1994. **62**(3): p. 1058-63.

320. Taylor, A.P. and H.W. Murray, *Intracellular antimicrobial activity in the absence of interferon-gamma: effect of interleukin-12 in experimental visceral leishmaniasis in interferon-gamma gene-disrupted mice.* J Exp Med, 1997. **185**(7): p. 1231-9.
321. Nylen, S. and D. Sacks, *Interleukin-10 and the pathogenesis of human visceral leishmaniasis.* Trends Immunol, 2007. **28**(9): p. 378-84.
322. Bradley, D.J., *Regulation of Leishmania populations within the host. II. genetic control of acute susceptibility of mice to Leishmania donovani infection.* Clin Exp Immunol, 1977. **30**(1): p. 130-40.
323. Gruenheid, S., et al., *Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome.* J Exp Med, 1997. **185**(4): p. 717-30.
324. Vidal, S., et al., *The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene.* J Exp Med, 1995. **182**(3): p. 655-66.
325. Crocker, P.R., J.M. Blackwell, and D.J. Bradley, *Expression of the natural resistance gene Lsh in resident liver macrophages.* Infect Immun, 1984. **43**(3): p. 1033-40.
326. Fritsche, G., et al., *Nramp1 functionality increases inducible nitric oxide synthase transcription via stimulation of IFN regulatory factor 1 expression.* J Immunol, 2003. **171**(4): p. 1994-8.
327. White, J.K., et al., *Slc11a1-mediated resistance to Salmonella enterica serovar Typhimurium and Leishmania donovani infections does not require functional inducible nitric oxide synthase or phagocyte oxidase activity.* J Leukoc Biol, 2005. **77**(3): p. 311-20.
328. Stober, C.B., et al., *Slc11a1, formerly Nramp1, is expressed in dendritic cells and influences major histocompatibility complex class II expression and antigen-presenting cell function.* Infect Immun, 2007. **75**(10): p. 5059-67.
329. Kaye, P.M. and J.M. Blackwell, *Lsh, antigen presentation and the development of CMI.* Res Immunol, 1989. **140**(8): p. 810-5; discussion 815-22.
330. Blackwell, J.M., et al., *Understanding the multiple functions of Nramp1.* Microbes Infect, 2000. **2**(3): p. 317-21.
331. Mohamed, H.S., et al., *SLC11A1 (formerly NRAMP1) and susceptibility to visceral leishmaniasis in The Sudan.* Eur J Hum Genet, 2004. **12**(1): p. 66-74.
332. Blackwell, J.M., et al., *SLC11A1 (formerly NRAMP1) and disease resistance.* Cell Microbiol, 2001. **3**(12): p. 773-84.
333. Peters, N.C., et al., *In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies.* Science, 2008. **321**(5891): p. 970-4.
334. Laskay, T., G. van Zandbergen, and W. Solbach, *Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor.* Immunobiology, 2008. **213**(3-4): p. 183-91.
335. Laskay, T., G. van Zandbergen, and W. Solbach, *Neutrophil granulocytes--Trojan horses for Leishmania major and other intracellular microbes?* Trends Microbiol, 2003. **11**(5): p. 210-4.
336. Ng, L.G., et al., *Migratory dermal dendritic cells act as rapid sensors of protozoan parasites.* PLoS Pathog, 2008. **4**(11): p. e1000222.
337. Hawn, T.R., et al., *Leishmania major activates IL-1 alpha expression in macrophages through a MyD88-dependent pathway.* Microbes Infect, 2002. **4**(8): p. 763-71.
338. de Veer, M.J., et al., *MyD88 is essential for clearance of Leishmania major: possible role for lipophosphoglycan and Toll-like receptor 2 signaling.* Eur J Immunol, 2003. **33**(10): p. 2822-31.
339. Muraille, E., et al., *Genetically resistant mice lacking MyD88-adapter protein display a high susceptibility to Leishmania major infection associated with a polarized Th2 response.* J Immunol, 2003. **170**(8): p. 4237-41.
340. Revaz-Breton, M., et al., *The MyD88 protein 88 pathway is differently involved in immune responses induced by distinct substrains of Leishmania major.* Eur J Immunol, 2010. **40**(6): p. 1697-707.

341. De Trez, C., et al., *Myd88-dependent in vivo maturation of splenic dendritic cells induced by Leishmania donovani and other Leishmania species.* Infect Immun, 2004. **72**(2): p. 824-32.
342. Vargas-Inchaustegui, D.A., et al., *Distinct roles for MyD88 and Toll-like receptor 2 during Leishmania braziliensis infection in mice.* Infect Immun, 2009. **77**(7): p. 2948-56.
343. Qureshi, S.T., et al., *Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4).* J Exp Med, 1999. **189**(4): p. 615-25.
344. Hoshino, K., et al., *Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product.* J Immunol, 1999. **162**(7): p. 3749-52.
345. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene.* Science, 1998. **282**(5396): p. 2085-8.
346. Kropf, P., et al., *Infection of C57BL/10ScCr and C57BL/10ScNCr mice with Leishmania major reveals a role for Toll-like receptor 4 in the control of parasite replication.* J Leukoc Biol, 2004. **76**(1): p. 48-57.
347. Kropf, P., et al., *Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite Leishmania major.* Infect Immun, 2004. **72**(4): p. 1920-8.
348. Ribeiro-Gomes, F.L., et al., *Neutrophils activate macrophages for intracellular killing of Leishmania major through recruitment of TLR4 by neutrophil elastase.* J Immunol, 2007. **179**(6): p. 3988-94.
349. Whitaker, S.M., et al., *Leishmania pifanoi proteoglycolipid complex P8 induces macrophage cytokine production through Toll-like receptor 4.* Infect Immun, 2008. **76**(5): p. 2149-56.
350. Abou Fakher, F.H., et al., *TLR9-dependent activation of dendritic cells by DNA from Leishmania major favors Th1 cell development and the resolution of lesions.* J Immunol, 2009. **182**(3): p. 1386-96.
351. Liese, J., U. Schleicher, and C. Bogdan, *TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis.* Eur J Immunol, 2007. **37**(12): p. 3424-34.
352. Martinez-Salazar, B., M. Berzunza-Cruz, and I. Becker, *[Leishmania mexicana DNA activates murine macrophages and increases their TLR9 expression].* Gac Med Mex, 2008. **144**(2): p. 99-104.
353. Schleicher, U., et al., *NK cell activation in visceral leishmaniasis requires TLR9, myeloid DCs, and IL-12, but is independent of plasmacytoid DCs.* J Exp Med, 2007. **204**(4): p. 893-906.
354. Lang, T., et al., *Leishmania donovani-infected macrophages: characterization of the parasitophorous vacuole and potential role of this organelle in antigen presentation.* J Cell Sci, 1994. **107 (Pt 8)**: p. 2137-50.
355. Fruth, U., N. Solioz, and J.A. Louis, *Leishmania major interferes with antigen presentation by infected macrophages.* J Immunol, 1993. **150**(5): p. 1857-64.
356. Prina, E., et al., *Antigen presentation capacity of murine macrophages infected with Leishmania amazonensis amastigotes.* J Immunol, 1993. **151**(4): p. 2050-61.
357. Reiner, N.E., W. Ng, and W.R. McMaster, *Parasite-accessory cell interactions in murine leishmaniasis. II. Leishmania donovani suppresses macrophage expression of class I and class II major histocompatibility complex gene products.* J Immunol, 1987. **138**(6): p. 1926-32.
358. Garcia, M.R., et al., *Epitope cleavage by Leishmania endopeptidase(s) limits the efficiency of the exogenous pathway of major histocompatibility complex class I-associated antigen presentation.* Eur J Immunol, 1997. **27**(4): p. 1005-13.
359. Kima, P.E., et al., *Leishmania-infected macrophages sequester endogenously synthesized parasite antigens from presentation to CD4+ T cells.* Eur J Immunol, 1996. **26**(12): p. 3163-9.
360. Wolfram, M., et al., *Antigen presentation by Leishmania mexicana-infected macrophages: activation of helper T cells specific for amastigote cysteine proteinases requires intracellular killing of the parasites.* Eur J Immunol, 1995. **25**(4): p. 1094-100.

361. Engelhorn, S., A. Bruckner, and H.G. Remold, *A soluble factor produced by inoculation of human monocytes with Leishmania donovani promastigotes suppresses IFN-gamma-dependent monocyte activation.* J Immunol, 1990. **145**(8): p. 2662-8.
362. Wolfram, M., et al., *Antigen presentation by Leishmania mexicana-infected macrophages: activation of helper T cells by a model parasite antigen secreted into the parasitophorous vacuole or expressed on the amastigote surface.* Eur J Immunol, 1996. **26**(12): p. 3153-62.
363. Prickett, S., et al., *In vivo recognition of ovalbumin expressed by transgenic Leishmania is determined by its subcellular localization.* J Immunol, 2006. **176**(8): p. 4826-33.
364. Bennett, C.L., et al., *Uncompromised generation of a specific H-2DM-dependent peptide-MHC class II complex from exogenous antigen in Leishmania mexicana-infected dendritic cells.* Eur J Immunol, 2003. **33**(12): p. 3504-13.
365. Konecny, P., et al., *Murine dendritic cells internalize Leishmania major promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation in vitro.* Eur J Immunol, 1999. **29**(6): p. 1803-11.
366. Ghosh, M., et al., *Leishmania donovani infection of human myeloid dendritic cells leads to a Th1 response in CD4+ T cells from healthy donors and patients with kala-azar.* J Infect Dis, 2006. **194**(3): p. 294-301.
367. Murray, H.W., et al., *Requirement for T cells and effect of lymphokines in successful chemotherapy for an intracellular infection. Experimental visceral leishmaniasis.* J Clin Invest, 1989. **83**(4): p. 1253-7.
368. Murray, H.W. and S. Delph-Etienne, *Roles of endogenous gamma interferon and macrophage microbicidal mechanisms in host response to chemotherapy in experimental visceral leishmaniasis.* Infect Immun, 2000. **68**(1): p. 288-93.
369. Sundar, S., F. Rosenkaimer, and H.W. Murray, *Successful treatment of refractory visceral leishmaniasis in India using antimony plus interferon-gamma.* J Infect Dis, 1994. **170**(3): p. 659-62.
370. Badaro, R., et al., *Treatment of visceral leishmaniasis with pentavalent antimony and interferon gamma.* N Engl J Med, 1990. **322**(1): p. 16-21.
371. Squires, K.E., et al., *Immunochemotherapy for visceral leishmaniasis: a controlled pilot trial of antimony versus antimony plus interferon-gamma.* Am J Trop Med Hyg, 1993. **48**(5): p. 666-9.
372. Murray, H.W., et al., *Immunoenhancement combined with amphotericin B as treatment for experimental visceral leishmaniasis.* Antimicrob Agents Chemother, 2003. **47**(8): p. 2513-7.
373. Murray, H.W., et al., *Interleukin-12 regulates the response to chemotherapy in experimental visceral Leishmaniasis.* J Infect Dis, 2000. **182**(5): p. 1497-502.
374. Murray, H.W., *Interleukin 10 receptor blockade--pentavalent antimony treatment in experimental visceral leishmaniasis.* Acta Trop, 2005. **93**(3): p. 295-301.
375. Zubairi, S., et al., *Immunotherapy with OX40L-Fc or anti-CTLA-4 enhances local tissue responses and killing of Leishmania donovani.* Eur J Immunol, 2004. **34**(5): p. 1433-40.
376. Murphy, M.L., et al., *Blockade of CTLA-4 enhances host resistance to the intracellular pathogen, Leishmania donovani.* J Immunol, 1998. **161**(8): p. 4153-60.
377. Murphy, M.L., et al., *B7-2 blockade enhances T cell responses to Leishmania donovani.* J Immunol, 1997. **159**(9): p. 4460-6.
378. Joshi, T., et al., *B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against Leishmania donovani infections.* PLoS Pathog, 2009. **5**(5): p. e1000431.
379. Haque, A., et al., *Therapeutic glucocorticoid-induced TNF receptor-mediated amplification of CD4+ T cell responses enhances antiparasitic immunity.* J Immunol, 2010. **184**(5): p. 2583-92.
380. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development.* Nat Rev Immunol, 2007. **7**(1): p. 19-30.
381. Liu, K. and M.C. Nussenzweig, *Origin and development of dendritic cells.* Immunol Rev, 2010. **234**(1): p. 45-54.
382. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells.* Science, 2010. **327**(5966): p. 656-61.

383. Nickol, A.D. and P.F. Bonventre, *Immunosuppression associated with visceral leishmaniasis of hamsters.* Parasite Immunol, 1985. **7**(4): p. 439-49.
384. Nickol, A.D. and P.F. Bonventre, *Visceral leishmaniasis in congenic mice of susceptible and resistant phenotypes: immunosuppression by adherent spleen cells.* Infect Immun, 1985. **50**(1): p. 160-8.
385. Heinzel, F.P., et al., *Production of interferon gamma, interleukin 2, interleukin 4, and interleukin 10 by CD4+ lymphocytes in vivo during healing and progressive murine leishmaniasis.* Proc Natl Acad Sci U S A, 1991. **88**(16): p. 7011-5.
386. Ghalib, H.W., et al., *Interleukin 10 production correlates with pathology in human Leishmania donovani infections.* J Clin Invest, 1993. **92**(1): p. 324-9.
387. Karp, C.L., et al., *In vivo cytokine profiles in patients with kala-azar. Marked elevation of both interleukin-10 and interferon-gamma.* J Clin Invest, 1993. **91**(4): p. 1644-8.
388. Murphy, M.L., et al., *IL-10 mediates susceptibility to Leishmania donovani infection.* Eur J Immunol, 2001. **31**(10): p. 2848-56.
389. Murray, H.W., et al., *Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy.* Infect Immun, 2002. **70**(11): p. 6284-93.
390. Nylen, S., et al., *Splenic accumulation of IL-10 mRNA in T cells distinct from CD4+CD25+ (Foxp3) regulatory T cells in human visceral leishmaniasis.* J Exp Med, 2007. **204**(4): p. 805-17.
391. Stager, S., et al., *Distinct roles for IL-6 and IL-12p40 in mediating protection against Leishmania donovani and the expansion of IL-10+ CD4+ T cells.* Eur J Immunol, 2006. **36**(7): p. 1764-71.
392. Maroof, A., et al., *Posttranscriptional regulation of il10 gene expression allows natural killer cells to express immunoregulatory function.* Immunity, 2008. **29**(2): p. 295-305.
393. Chandra, D. and S. Naik, *Leishmania donovani infection down-regulates TLR2-stimulated IL-12p40 and activates IL-10 in cells of macrophage/monocytic lineage by modulating MAPK pathways through a contact-dependent mechanism.* Clin Exp Immunol, 2008. **154**(2): p. 224-34.
394. Haldar, A.K., et al., *Leishmania donovani Isolates with Antimony-Resistant but Not -Sensitive Phenotype Inhibit Sodium Antimony Gluconate-Induced Dendritic Cell Activation.* PLoS Pathog, 2010. **6**(5): p. e1000907.
395. Carrera, L., et al., *Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice.* J Exp Med, 1996. **183**(2): p. 515-26.
396. Liu, Y., et al., *Heat-stable antigen is a costimulatory molecule for CD4 T cell growth.* J Exp Med, 1992. **175**(2): p. 437-45.
397. Saha, B., et al., *Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory molecules on Leishmania-infected macrophages and its implication in the suppression of cell-mediated immunity.* Eur J Immunol, 1995. **25**(9): p. 2492-8.
398. Kaye, P.M., et al., *Deficient expression of co-stimulatory molecules on Leishmania-infected macrophages.* Eur J Immunol, 1994. **24**(11): p. 2850-4.
399. De Almeida, M.C., S.A. Cardoso, and M. Barral-Netto, *Leishmania (Leishmania) chagasi infection alters the expression of cell adhesion and costimulatory molecules on human monocyte and macrophage.* Int J Parasitol, 2003. **33**(2): p. 153-62.
400. Xin, L., K. Li, and L. Soong, *Down-regulation of dendritic cell signaling pathways by Leishmania amazonensis amastigotes.* Mol Immunol, 2008. **45**(12): p. 3371-82.
401. Boggiatto, P.M., et al., *Altered dendritic cell phenotype in response to Leishmania amazonensis amastigote infection is mediated by MAP kinase, ERK.* Am J Pathol, 2009. **174**(5): p. 1818-26.
402. Favali, C., et al., *Leishmania amazonensis infection impairs differentiation and function of human dendritic cells.* J Leukoc Biol, 2007. **82**(6): p. 1401-6.
403. Carvalho, L.P., E.J. Pearce, and P. Scott, *Functional dichotomy of dendritic cells following interaction with Leishmania braziliensis: infected cells produce high levels of TNF-alpha, whereas bystander dendritic cells are activated to promote T cell responses.* J Immunol, 2008. **181**(9): p. 6473-80.

404. Ato, M., et al., *Loss of dendritic cell migration and impaired resistance to Leishmania donovani infection in mice deficient in CCL19 and CCL21.* J Immunol, 2006. **176**(9): p. 5486-93.
405. Maroof, A., et al., *Dendritic cells matured by inflammation induce CD86-dependent priming of naive CD8+ T cells in the absence of their cognate peptide antigen.* J Immunol, 2009. **183**(11): p. 7095-103.
406. Brown, J.A., et al., *Blockade of CD86 ameliorates Leishmania major infection by down-regulating the Th2 response.* J Infect Dis, 1996. **174**(6): p. 1303-8.
407. Jebbari, H., et al., *Leishmania major promastigotes inhibit dendritic cell motility in vitro.* Infect Immun, 2002. **70**(2): p. 1023-6.
408. Ponte-Sucre, A., D. Heise, and H. Moll, *Leishmania major lipophosphoglycan modulates the phenotype and inhibits migration of murine Langerhans cells.* Immunology, 2001. **104**(4): p. 462-7.
409. Steigerwald, M. and H. Moll, *Leishmania major modulates chemokine and chemokine receptor expression by dendritic cells and affects their migratory capacity.* Infect Immun, 2005. **73**(4): p. 2564-7.
410. Tejle, K., et al., *Wild-type Leishmania donovani promastigotes block maturation, increase integrin expression and inhibit detachment of human monocyte-derived dendritic cells--the influence of phosphoglycans.* FEMS Microbiol Lett, 2008. **279**(1): p. 92-102.
411. Hsieh, C.S., et al., *Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages.* Science, 1993. **260**(5107): p. 547-9.
412. de Waal Malefyt, R., et al., *Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.* J Exp Med, 1991. **174**(5): p. 1209-20.
413. Wang, P., et al., *IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells.* J Immunol, 1994. **153**(2): p. 811-6.
414. Ranatunga, D., et al., *A human IL10 BAC transgene reveals tissue-specific control of IL-10 expression and alters disease outcome.* Proc Natl Acad Sci U S A, 2009. **106**(40): p. 17123-8.
415. Couper, K.N., et al., *IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection.* PLoS Pathog, 2008. **4**(2): p. e1000004.
416. Haringer, B., et al., *Identification and characterization of IL-10/IFN-gamma-producing effector-like T cells with regulatory function in human blood.* J Exp Med, 2009. **206**(5): p. 1009-17.
417. Nagase, H., et al., *Despite increased CD4+Foxp3+ cells within the infection site, BALB/c IL-4 receptor-deficient mice reveal CD4+Foxp3-negative T cells as a source of IL-10 in Leishmania major susceptibility.* J Immunol, 2007. **179**(4): p. 2435-44.
418. Suffia, I.J., et al., *Infected site-restricted Foxp3+ natural regulatory T cells are specific for microbial antigens.* J Exp Med, 2006. **203**(3): p. 777-88.
419. Mendez, S., et al., *Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity.* J Exp Med, 2004. **200**(2): p. 201-10.
420. Belkaid, Y., et al., *CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity.* Nature, 2002. **420**(6915): p. 502-7.
421. Kemp, K., et al., *Leishmania-specific T cells expressing interferon-gamma (IFN-gamma) and IL-10 upon activation are expanded in individuals cured of visceral leishmaniasis.* Clin Exp Immunol, 1999. **116**(3): p. 500-4.
422. Pohl-Koppe, A., et al., *Identification of a T cell subset capable of both IFN-gamma and IL-10 secretion in patients with chronic Borrelia burgdorferi infection.* J Immunol, 1998. **160**(4): p. 1804-10.
423. Gerosa, F., et al., *CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients.* Clin Immunol, 1999. **92**(3): p. 224-34.
424. Sakaguchi, S., et al., *Regulatory T cells and immune tolerance.* Cell, 2008. **133**(5): p. 775-87.

425. Maurya, R., et al., *Human visceral leishmaniasis is not associated with expansion or accumulation of Foxp3(+) CD4 cells in blood or spleen.* Parasite Immunol, 2010. **32**(7): p. 479-83.
426. Ganguly, S., et al., *Enhanced lesional Foxp3 expression and peripheral anergic lymphocytes indicate a role for regulatory T cells in Indian post-kala-azar dermal leishmaniasis.* J Invest Dermatol, 2010. **130**(4): p. 1013-22.
427. Pene, F., et al., *TLR2 and TLR4 contribute to sepsis-induced depletion of spleen dendritic cells.* Infect Immun, 2009.
428. Efron, P.A., et al., *Characterization of the systemic loss of dendritic cells in murine lymph nodes during polymicrobial sepsis.* J Immunol, 2004. **173**(5): p. 3035-43.
429. Sundquist, M. and M.J. Wick, *Salmonella induces death of CD8alpha(+) dendritic cells but not CD11c(int)CD11b(+) inflammatory cells in vivo via MyD88 and TNFR1.* J Leukoc Biol, 2009. **85**(2): p. 225-34.
430. Serbina, N.V., et al., *TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection.* Immunity, 2003. **19**(1): p. 59-70.
431. Kamanaka, M., et al., *Protective role of CD40 in Leishmania major infection at two distinct phases of cell-mediated immunity.* Immunity, 1996. **4**(3): p. 275-81.
432. Campbell, K.A., et al., *CD40 ligand is required for protective cell-mediated immunity to Leishmania major.* Immunity, 1996. **4**(3): p. 283-9.
433. Martin, S., et al., *CD40 expression levels modulate regulatory T cells in Leishmania donovani infection.* J Immunol, 2010. **185**(1): p. 551-9.
434. Padigel, U.M. and J.P. Farrell, *CD40-CD40 ligand costimulation is not required for initiation and maintenance of a Th1-type response to Leishmania major infection.* Infect Immun, 2003. **71**(3): p. 1389-95.
435. Padigel, U.M., P.J. Perrin, and J.P. Farrell, *The development of a Th1-type response and resistance to Leishmania major infection in the absence of CD40-CD40L costimulation.* J Immunol, 2001. **167**(10): p. 5874-9.
436. Padigel, U.M., et al., *TRANCE-RANK costimulation is required for IL-12 production and the initiation of a Th1-type response to Leishmania major infection in CD40L-deficient mice.* J Immunol, 2003. **171**(10): p. 5437-41.
437. Soares, H., et al., *A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo.* J Exp Med, 2007. **204**(5): p. 1095-106.
438. Nolte, M.A., et al., *B cells are crucial for both development and maintenance of the splenic marginal zone.* J Immunol, 2004. **172**(6): p. 3620-7.
439. Shen, T., et al., *Increased PD-L1 expression and PD-L1/CD86 ratio on dendritic cells were associated with impaired dendritic cells function in HCV infection.* J Med Virol, 2010. **82**(7): p. 1152-9.
440. Kuipers, H., et al., *Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic cell-mediated CD4+ T cell activation.* Eur J Immunol, 2006. **36**(9): p. 2472-82.
441. Saha, S., et al., *IL-10- and TGF-beta-mediated susceptibility in kala-azar and post-kala-azar dermal leishmaniasis: the significance of amphotericin B in the control of Leishmania donovani infection in India.* J Immunol, 2007. **179**(8): p. 5592-603.
442. Gomes, N.A., et al., *TGF-beta mediates CTLA-4 suppression of cellular immunity in murine kalaazar.* J Immunol, 2000. **164**(4): p. 2001-8.
443. Wilson, M.E., et al., *The importance of TGF-beta in murine visceral leishmaniasis.* J Immunol, 1998. **161**(11): p. 6148-55.
444. Rodrigues, O.R., et al., *H-2 complex influences cytokine gene expression in Leishmania infantum-infected macrophages.* Cell Immunol, 2006. **243**(2): p. 118-26.
445. Pitta, M.G., et al., *IL-17 and IL-22 are associated with protection against human kala azar caused by Leishmania donovani.* J Clin Invest, 2009. **119**(8): p. 2379-87.
446. Jayakumar, A., et al., *Transcriptional inhibition of interleukin-12 promoter activity in Leishmania spp.-infected macrophages.* J Parasitol, 2008. **94**(1): p. 84-93.
447. Cameron, P., et al., *Inhibition of lipopolysaccharide-induced macrophage IL-12 production by Leishmania mexicana amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway.* J Immunol, 2004. **173**(5): p. 3297-304.

448. Feng, G.J., et al., *Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: Leishmania phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase.* J Immunol, 1999. **163**(12): p. 6403-12.
449. Weinheber, N., et al., *Phagocytosis of Leishmania mexicana amastigotes by macrophages leads to a sustained suppression of IL-12 production.* Eur J Immunol, 1998. **28**(8): p. 2467-77.
450. Liu, D., et al., *Leishmania major phosphoglycans influence the host early immune response by modulating dendritic cell functions.* Infect Immun, 2009. **77**(8): p. 3272-83.
451. von Stebut, E., et al., *Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-Leishmania immunity.* J Exp Med, 1998. **188**(8): p. 1547-52.
452. von Stebut, E., et al., *Leishmania major-infected murine langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous Leishmaniasis.* Eur J Immunol, 2000. **30**(12): p. 3498-506.
453. McDowell, M.A., et al., *Leishmania priming of human dendritic cells for CD40 ligand-induced interleukin-12p70 secretion is strain and species dependent.* Infect Immun, 2002. **70**(8): p. 3994-4001.
454. Gorak, P.M., C.R. Engwerda, and P.M. Kaye, *Dendritic cells, but not macrophages, produce IL-12 immediately following Leishmania donovani infection.* Eur J Immunol, 1998. **28**(2): p. 687-95.
455. Quinones, M., et al., *Preformed membrane-associated stores of interleukin (IL)-12 are a previously unrecognized source of bioactive IL-12 that is mobilized within minutes of contact with an intracellular parasite.* J Exp Med, 2000. **192**(4): p. 507-16.
456. Stanley, A.C., et al., *VCAM-1 and VLA-4 modulate dendritic cell IL-12p40 production in experimental visceral leishmaniasis.* PLoS Pathog, 2008. **4**(9): p. e1000158.
457. Satoskar, A.R., et al., *IL-12 gene-deficient C57BL/6 mice are susceptible to Leishmania donovani but have diminished hepatic immunopathology.* Eur J Immunol, 2000. **30**(3): p. 834-9.
458. Murray, H.W., *Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis.* J Infect Dis, 1997. **175**(6): p. 1477-9.
459. Murray, H.W. and J. Hariprashad, *Interleukin 12 is effective treatment for an established systemic intracellular infection: experimental visceral leishmaniasis.* J Exp Med, 1995. **181**(1): p. 387-91.
460. Berberich, C., et al., *Dendritic cell (DC)-based protection against an intracellular pathogen is dependent upon DC-derived IL-12 and can be induced by molecularly defined antigens.* J Immunol, 2003. **170**(6): p. 3171-9.
461. Descoteaux, A., et al., *Leishmania donovani lipophosphoglycan selectively inhibits signal transduction in macrophages.* J Immunol, 1991. **146**(8): p. 2747-53.
462. Ghalib, H.W., et al., *IL-12 enhances Th1-type responses in human Leishmania donovani infections.* J Immunol, 1995. **154**(9): p. 4623-9.
463. Mathur, R.K., et al., *Reciprocal CD40 signals through p38MAPK and ERK-1/2 induce counteracting immune responses.* Nat Med, 2004. **10**(5): p. 540-4.
464. Awasthi, A., et al., *CD40 signaling is impaired in L. major-infected macrophages and is rescued by a p38MAPK activator establishing a host-protective memory T cell response.* J Exp Med, 2003. **197**(8): p. 1037-43.
465. Rub, A., et al., *Cholesterol depletion associated with Leishmania major infection alters macrophage CD40 signalosome composition and effector function.* Nat Immunol, 2009. **10**(3): p. 273-80.
466. Tuettenberg, A., et al., *CD40 signalling induces IL-10-producing, tolerogenic dendritic cells.* Exp Dermatol, 2010. **19**(1): p. 44-53.
467. Yang, Z., D.M. Mosser, and X. Zhang, *Activation of the MAPK, ERK, following Leishmania amazonensis infection of macrophages.* J Immunol, 2007. **178**(2): p. 1077-85.

468. Ghosh, S., et al., *Leishmania donovani suppresses activated protein 1 and NF-kappaB activation in host macrophages via ceramide generation: involvement of extracellular signal-regulated kinase.* Infect Immun, 2002. **70**(12): p. 6828-38.
469. Ghosh, S., et al., *Generation of ceramide in murine macrophages infected with Leishmania donovani alters macrophage signaling events and aids intracellular parasitic survival.* Mol Cell Biochem, 2001. **223**(1-2): p. 47-60.
470. Prive, C. and A. Descoteaux, *Leishmania donovani promastigotes evade the activation of mitogen-activated protein kinases p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase-1/2 during infection of naive macrophages.* Eur J Immunol, 2000. **30**(8): p. 2235-44.
471. Saar, Y., et al., *Characterization of developmentally-regulated activities in axenic amastigotes of Leishmania donovani.* Mol Biochem Parasitol, 1998. **95**(1): p. 9-20.
472. Agrawal, S., et al., *Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos.* J Immunol, 2003. **171**(10): p. 4984-9.
473. Chau, T.A., et al., *Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome.* Nat Med, 2009. **15**(6): p. 641-8.
474. Kaji, R., et al., *Bacterial teichoic acids reverse predominant IL-12 production induced by certain lactobacillus strains into predominant IL-10 production via TLR2-dependent ERK activation in macrophages.* J Immunol, 2010. **184**(7): p. 3505-13.
475. Caparros, E., et al., *DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production.* Blood, 2006. **107**(10): p. 3950-8.
476. Geijtenbeek, T.B., et al., *Mycobacteria target DC-SIGN to suppress dendritic cell function.* J Exp Med, 2003. **197**(1): p. 7-17.
477. Colmenares, M., et al., *Dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN, CD209), a C-type surface lectin in human DCs, is a receptor for Leishmania amastigotes.* J Biol Chem, 2002. **277**(39): p. 36766-9.
478. Appelmelk, B.J., et al., *Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells.* J Immunol, 2003. **170**(4): p. 1635-9.
479. Colmenares, M., et al., *The dendritic cell receptor DC-SIGN discriminates among species and life cycle forms of Leishmania.* J Immunol, 2004. **172**(2): p. 1186-90.
480. Caminschi, I., et al., *Functional comparison of mouse CIRE/mouse DC-SIGN and human DC-SIGN.* Int Immunol, 2006. **18**(5): p. 741-53.
481. Becker, I., et al., *Leishmania lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2.* Mol Biochem Parasitol, 2003. **130**(2): p. 65-74.
482. Flandin, J.F., F. Chano, and A. Descoteaux, *RNA interference reveals a role for TLR2 and TLR3 in the recognition of Leishmania donovani promastigotes by interferon-gamma-primed macrophages.* Eur J Immunol, 2006. **36**(2): p. 411-20.
483. Kavoosi, G., S.K. Ardestani, and A. Kariminia, *The involvement of TLR2 in cytokine and reactive oxygen species (ROS) production by PBMCs in response to Leishmania major phosphoglycans (PGs).* Parasitology, 2009. **136**(10): p. 1193-9.
484. Guerra, C.S., et al., *Histopathological analysis of initial cellular response in TLR-2 deficient mice experimentally infected by Leishmania (L.) amazonensis.* Int J Exp Pathol, 2010.
485. Silvestre, R., et al., *The contribution of Toll-like receptor 2 to the innate recognition of a Leishmania infantum silent information regulator 2 protein.* Immunology, 2009. **128**(4): p. 484-99.
486. Anderson, C.F., et al., *IL-27 Regulates IL-10 and IL-17 from CD4+ Cells in Nonhealing Leishmania major Infection.* J Immunol, 2009.
487. Levings, M.K., et al., *Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells.* Blood, 2005. **105**(3): p. 1162-9.

488. Saraiva, M., et al., *Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose.* Immunity, 2009. **31**(2): p. 209-19.
489. Gabrysova, L., et al., *Negative feedback control of the autoimmune response through antigen-induced differentiation of IL-10-secreting Th1 cells.* J Exp Med, 2009. **206**(8): p. 1755-67.
490. Pulendran, B., H. Tang, and S. Manicassamy, *Programming dendritic cells to induce T(H)2 and tolerogenic responses.* Nat Immunol, 2010. **11**(8): p. 647-55.
491. Demangel, C., P. Bertolino, and W.J. Britton, *Autocrine IL-10 impairs dendritic cell (DC)-derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL-12 production.* Eur J Immunol, 2002. **32**(4): p. 994-1002.
492. Boonstra, A., et al., *Macrophages and myeloid dendritic cells, but not plasmacytoid dendritic cells, produce IL-10 in response to MyD88- and TRIF-dependent TLR signals, and TLR-independent signals.* J Immunol, 2006. **177**(11): p. 7551-8.
493. Xia, C.Q. and K.J. Kao, *Suppression of interleukin-12 production through endogenously secreted interleukin-10 in activated dendritic cells: involvement of activation of extracellular signal-regulated protein kinase.* Scand J Immunol, 2003. **58**(1): p. 23-32.
494. Kajino, K., et al., *Involvement of IL-10 in exhaustion of myeloid dendritic cells and rescue by CD40 stimulation.* Immunology, 2007. **120**(1): p. 28-37.
495. Wang, S., et al., *Augmentation of antigen-presenting and Th1-promoting functions of dendritic cells by WSX-1(IL-27R) deficiency.* J Immunol, 2007. **179**(10): p. 6421-8.
496. Holscher, C., et al., *The IL-27 receptor chain WSX-1 differentially regulates antibacterial immunity and survival during experimental tuberculosis.* J Immunol, 2005. **174**(6): p. 3534-44.
497. Pirhonen, J., et al., *IFN-alpha regulates Toll-like receptor-mediated IL-27 gene expression in human macrophages.* J Leukoc Biol, 2007. **82**(5): p. 1185-92.
498. Remoli, M.E., et al., *IFN-beta modulates the response to TLR stimulation in human DC: involvement of IFN regulatory factor-1 (IRF-1) in IL-27 gene expression.* Eur J Immunol, 2007. **37**(12): p. 3499-508.
499. Molle, C., et al., *IL-27 synthesis induced by TLR ligation critically depends on IFN regulatory factor 3.* J Immunol, 2007. **178**(12): p. 7607-15.
500. Molle, C., M. Goldman, and S. Goriely, *Critical role of the IFN-stimulated gene factor 3 complex in TLR-mediated IL-27p28 gene expression revealing a two-step activation process.* J Immunol, 2010. **184**(4): p. 1784-92.
501. Zhang, J., et al., *Activation of IL-27 p28 gene transcription by interferon regulatory factor 8 in cooperation with interferon regulatory factor1.* J Biol Chem, 2010.
502. Phillips, R., et al., *Innate killing of Leishmania donovani by macrophages of the splenic marginal zone requires IRF-7.* PLoS Pathog, 2010. **6**(3): p. e1000813.
503. Jung, S., et al., *In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens.* Immunity, 2002. **17**(2): p. 211-20.
504. Zaft, T., et al., *CD11chigh dendritic cell ablation impairs lymphopenia-driven proliferation of naive and memory CD8+ T cells.* J Immunol, 2005. **175**(10): p. 6428-35.
505. Hochweller, K., et al., *A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells.* Eur J Immunol, 2008. **38**(10): p. 2776-83.
506. Phythian-Adams, A.T., et al., *CD11c depletion severely disrupts Th2 induction and development in vivo.* J Exp Med, 2010. **207**(10): p. 2089-96.
507. Stranges, P.B., et al., *Elimination of antigen-presenting cells and autoreactive T cells by Fas contributes to prevention of autoimmunity.* Immunity, 2007. **26**(5): p. 629-41.
508. Buch, T., et al., *A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration.* Nat Methods, 2005. **2**(6): p. 419-26.
509. Kirby, A.C., et al., *SIGNR1-negative red pulp macrophages protect against acute streptococcal sepsis after Leishmania donovani-induced loss of marginal zone macrophages.* Am J Pathol, 2009. **175**(3): p. 1107-15.

510. Brewig, N., et al., *Priming of CD8+ and CD4+ T cells in experimental leishmaniasis is initiated by different dendritic cell subtypes.* J Immunol, 2009. **182**(2): p. 774-83.
511. Vremec, D., et al., *Factors determining the spontaneous activation of splenic dendritic cells in culture.* Innate Immun, 2010.
512. Bar-On, L. and S. Jung, *Defining dendritic cells by conditional and constitutive cell ablation.* Immunol Rev, 2010. **234**(1): p. 76-89.
513. Fleming, T.J., M.L. Fleming, and T.R. Malek, *Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family.* J Immunol, 1993. **151**(5): p. 2399-408.
514. D'Andrea, A., et al., *Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells.* J Exp Med, 1993. **178**(3): p. 1041-8.
515. De Smedt, T., et al., *Effect of interleukin-10 on dendritic cell maturation and function.* Eur J Immunol, 1997. **27**(5): p. 1229-35.
516. Buelens, C., et al., *Human dendritic cell responses to lipopolysaccharide and CD40 ligation are differentially regulated by interleukin-10.* Eur J Immunol, 1997. **27**(8): p. 1848-52.
517. Corinti, S., et al., *Regulatory activity of autocrine IL-10 on dendritic cell functions.* J Immunol, 2001. **166**(7): p. 4312-8.
518. Braun, M.C., E. Lahey, and B.L. Kelsall, *Selective suppression of IL-12 production by chemoattractants.* J Immunol, 2000. **164**(6): p. 3009-17.
519. Koski, G.K., L.A. Lyakh, and N.R. Rice, *Rapid lipopolysaccharide-induced differentiation of CD14(+) monocytes into CD83(+) dendritic cells is modulated under serum-free conditions by exogenously added IFN-gamma and endogenously produced IL-10.* Eur J Immunol, 2001. **31**(12): p. 3773-81.
520. Bullens, D.M., et al., *CD40L-induced IL-12 production is further enhanced by the Th2 cytokines IL-4 and IL-13.* Scand J Immunol, 2001. **53**(5): p. 455-63.
521. Carbonneil, C., et al., *Dendritic cells generated in the presence of interferon-alpha stimulate allogeneic CD4+ T-cell proliferation: modulation by autocrine IL-10, enhanced T-cell apoptosis and T regulatory type 1 cells.* Int Immunol, 2004. **16**(7): p. 1037-52.
522. Braun, M.C., et al., *Cholera toxin suppresses interleukin (IL)-12 production and IL-12 receptor beta1 and beta2 chain expression.* J Exp Med, 1999. **189**(3): p. 541-52.
523. Melillo, J.A., et al., *Dendritic cell (DC)-specific targeting reveals Stat3 as a negative regulator of DC function.* J Immunol, 2010. **184**(5): p. 2638-45.
524. Berlato, C., et al., *Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation.* J Immunol, 2002. **168**(12): p. 6404-11.
525. Re, F. and J.L. Strominger, *IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells.* J Immunol, 2004. **173**(12): p. 7548-55.
526. Igietseme, J.U., et al., *Suppression of endogenous IL-10 gene expression in dendritic cells enhances antigen presentation for specific Th1 induction: potential for cellular vaccine development.* J Immunol, 2000. **164**(8): p. 4212-9.
527. Jones, H.E., et al., *The differential response of human dendritic cells to live and killed Neisseria meningitidis.* Cell Microbiol, 2007. **9**(12): p. 2856-69.
528. Darrah, P.A., et al., *IL-10 production differentially influences the magnitude, quality, and protective capacity of Th1 responses depending on the vaccine platform.* J Exp Med, 2010. **207**(7): p. 1421-33.
529. Colino, J. and C.M. Snapper, *Opposing signals from pathogen-associated molecular patterns and IL-10 are critical for optimal dendritic cell induction of in vivo humoral immunity to Streptococcus pneumoniae.* J Immunol, 2003. **171**(7): p. 3508-19.
530. Chang, W.L., et al., *Exposure of myeloid dendritic cells to exogenous or endogenous IL-10 during maturation determines their longevity.* J Immunol, 2007. **178**(12): p. 7794-804.

531. Wirtz, S., et al., *Protection from lethal septic peritonitis by neutralizing the biological function of interleukin 27.* J Exp Med, 2006. **203**(8): p. 1875-81.
532. Kalliolias, G.D. and L.B. Ivashkiv, *IL-27 activates human monocytes via STAT1 and suppresses IL-10 production but the inflammatory functions of IL-27 are abrogated by TLRs and p38.* J Immunol, 2008. **180**(9): p. 6325-33.
533. Iyer, S.S., A.A. Ghaffari, and G. Cheng, *Lipopolysaccharide-Mediated IL-10 Transcriptional Regulation Requires Sequential Induction of Type I IFNs and IL-27 in Macrophages.* J Immunol, 2010. **185**(11): p. 6599-6607.
534. Assenmacher, M., J. Schmitz, and A. Radbruch, *Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-gamma and in interleukin-4-expressing cells.* Eur J Immunol, 1994. **24**(5): p. 1097-101.
535. Rosas, L.E., et al., *Interleukin-27R (WSX-1/T-cell cytokine receptor) gene-deficient mice display enhanced resistance to leishmania donovani infection but develop severe liver immunopathology.* Am J Pathol, 2006. **168**(1): p. 158-69.
536. Findlay, E.G., et al., *Essential role for IL-27 receptor signaling in prevention of Th1-mediated immunopathology during malaria infection.* J Immunol, 2010. **185**(4): p. 2482-92.
537. Chen, Y., et al., *Distinct response of liver myeloid dendritic cells to endotoxin is mediated by IL-27.* J Hepatol, 2009.
538. Robinson, C.M. and G.J. Nau, *Interleukin-12 and interleukin-27 regulate macrophage control of Mycobacterium tuberculosis.* J Infect Dis, 2008. **198**(3): p. 359-66.
539. Stumhofer, J.S., et al., *A role for IL-27p28 as an antagonist of gp130-mediated signaling.* Nat Immunol, 2010. **11**(12): p. 1119-1126.
540. Ruckerl, D., et al., *Alternatively activated macrophages express the IL-27 receptor alpha chain WSX-1.* Immunobiology, 2006. **211**(6-8): p. 427-36.
541. Villarino, A.V., et al., *Positive and negative regulation of the IL-27 receptor during lymphoid cell activation.* J Immunol, 2005. **174**(12): p. 7684-91.
542. Lohoff, M., et al., *Enhanced TCR-induced apoptosis in interferon regulatory factor 4-deficient CD4(+) Th cells.* J Exp Med, 2004. **200**(2): p. 247-53.
543. Tominaga, N., et al., *Development of Th1 and not Th2 immune responses in mice lacking IFN-regulatory factor-4.* Int Immunol, 2003. **15**(1): p. 1-10.
544. Lohoff, M., et al., *Deficiency in the transcription factor interferon regulatory factor (IRF)-2 leads to severely compromised development of natural killer and T helper type 1 cells.* J Exp Med, 2000. **192**(3): p. 325-36.
545. Lohoff, M., et al., *Interferon regulatory factor-1 is required for a T helper 1 immune response in vivo.* Immunity, 1997. **6**(6): p. 681-9.
546. Steinberg, C., et al., *The IFN regulatory factor 7-dependent type I IFN response is not essential for early resistance against murine cytomegalovirus infection.* Eur J Immunol, 2009. **39**(4): p. 1007-18.
547. Honda, K., et al., *IRF-7 is the master regulator of type-I interferon-dependent immune responses.* Nature, 2005. **434**(7034): p. 772-7.
548. Jayakumar, A., et al., *Leishmania major infection activates NF-kappaB and interferon regulatory factors 1 and 8 in human dendritic cells.* Infect Immun, 2008. **76**(5): p. 2138-48.
549. Matte, C. and A. Descoteaux, *Leishmania donovani amastigotes impair gamma interferon-induced STAT1alpha nuclear translocation by blocking the interaction between STAT1alpha and importin-alpha5.* Infect Immun, 2010. **78**(9): p. 3736-43.
550. Scharton-Kersten, T., et al., *Interferon consensus sequence binding protein-deficient mice display impaired resistance to intracellular infection due to a primary defect in interleukin 12 p40 induction.* J Exp Med, 1997. **186**(9): p. 1523-34.
551. Lohoff, M., et al., *Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4.* Proc Natl Acad Sci U S A, 2002. **99**(18): p. 11808-12.
552. Giese, N.A., et al., *Interferon (IFN) consensus sequence-binding protein, a transcription factor of the IFN regulatory factor family, regulates immune responses in vivo through control of interleukin 12 expression.* J Exp Med, 1997. **186**(9): p. 1535-46.

553. Taki, S., et al., *Multistage regulation of Th1-type immune responses by the transcription factor IRF-1.* Immunity, 1997. **6**(6): p. 673-9.
554. Tan, R.S., et al., *Differential interleukin-10 expression in interferon regulatory factor-1 deficient mice during Plasmodium berghei blood-stage infection.* Parasite Immunol, 2000. **22**(9): p. 425-35.
555. Mittrucker, H.W., et al., *Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function.* Science, 1997. **275**(5299): p. 540-3.
556. Holtschke, T., et al., *Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene.* Cell, 1996. **87**(2): p. 307-17.
557. Ahyi, A.N., et al., *IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines.* J Immunol, 2009. **183**(3): p. 1598-606.
558. Lee, C.G., et al., *A distal cis-regulatory element, CNS-9, controls NFAT1 and IRF4-mediated IL-10 gene activation in T helper cells.* Mol Immunol, 2009. **46**(4): p. 613-21.
559. Khan, I.A., et al., *Production of nitric oxide (NO) is not essential for protection against acute Toxoplasma gondii infection in IRF-1-/- mice.* J Immunol, 1996. **156**(2): p. 636-43.
560. Zammit, D.J., et al., *Dendritic cells maximize the memory CD8 T cell response to infection.* Immunity, 2005. **22**(5): p. 561-70.
561. Huleatt, J.W. and L. Lefrancois, *Antigen-driven induction of CD11c on intestinal intraepithelial lymphocytes and CD8+ T cells in vivo.* J Immunol, 1995. **154**(11): p. 5684-93.
562. Hebel, K., et al., *Plasma cell differentiation in T-independent type 2 immune responses is independent of CD11c(high) dendritic cells.* Eur J Immunol, 2006. **36**(11): p. 2912-9.
563. Landsman, L., C. Varol, and S. Jung, *Distinct differentiation potential of blood monocyte subsets in the lung.* J Immunol, 2007. **178**(4): p. 2000-7.
564. Iannacone, M., et al., *Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus.* Nature, 2010. **465**(7301): p. 1079-83.
565. Probst, H.C., et al., *Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells.* Clin Exp Immunol, 2005. **141**(3): p. 398-404.
566. Lucas, M., et al., *Dendritic cells prime natural killer cells by trans-presenting interleukin 15.* Immunity, 2007. **26**(4): p. 503-17.
567. Ohnmacht, C., et al., *Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity.* J Exp Med, 2009.
568. Esmann, L., et al., *Phagocytosis of apoptotic cells by neutrophil granulocytes: diminished proinflammatory neutrophil functions in the presence of apoptotic cells.* J Immunol, 2010. **184**(1): p. 391-400.
569. Dalton, J.E., et al., *Inhibition of receptor tyrosine kinases restores immunocompetence and improves immune-dependent chemotherapy against experimental leishmaniasis in mice.* J Clin Invest, 2010. **120**(4): p. 1204-16.
570. Riboldi, E., et al., *Cutting edge: proangiogenic properties of alternatively activated dendritic cells.* J Immunol, 2005. **175**(5): p. 2788-92.
571. Matte, C., et al., *Leishmania donovani-induced macrophages cyclooxygenase-2 and prostaglandin E2 synthesis.* Parasite Immunol, 2001. **23**(4): p. 177-84.
572. Reiner, N.E. and C.J. Malemud, *Arachidonic acid metabolism in murine leishmaniasis (Donovani): ex-vivo evidence for increased cyclooxygenase and 5-lipoxygenase activity in spleen cells.* Cell Immunol, 1984. **88**(2): p. 501-10.
573. Horst, A.K., et al., *CEACAM1+ myeloid cells control angiogenesis in inflammation.* Blood, 2009. **113**(26): p. 6726-36.
574. Kishuku, M., et al., *Expression of soluble vascular endothelial growth factor receptor-1 in human monocyte-derived mature dendritic cells contributes to their antiangiogenic property.* J Immunol, 2009. **183**(12): p. 8176-85.
575. Tussiwand, R., et al., *Inhibition of natural type I IFN-producing and dendritic cell development by a small molecule receptor tyrosine kinase inhibitor with Flt3 affinity.* J Immunol, 2005. **175**(6): p. 3674-80.

576. Liu, C.H., et al., *Cutting edge: dendritic cells are essential for in vivo IL-12 production and development of resistance against Toxoplasma gondii infection in mice.* J Immunol, 2006. **177**(1): p. 31-5.
577. Kassim, S.H., et al., *In vivo ablation of CD11c-positive dendritic cells increases susceptibility to herpes simplex virus type 1 infection and diminishes NK and T-cell responses.* J Virol, 2006. **80**(8): p. 3985-93.
578. Jung, S., et al., *In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens.* Immunity, 2002. **17**(2): p. 211-20.
579. Loof, T.G., et al., *The contribution of dendritic cells to host defenses against Streptococcus pyogenes.* J Infect Dis, 2007. **196**(12): p. 1794-803.
580. Rahman, S., et al., *Depletion of dendritic cells enhances susceptibility to cell-free infection of human T cell leukemia virus type 1 in CD11c-diphtheria toxin receptor transgenic mice.* J Immunol, 2010. **184**(10): p. 5553-61.
581. Tian, T., et al., *In vivo depletion of CD11c+ cells delays the CD4+ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection.* J Immunol, 2005. **175**(5): p. 3268-72.
582. Levings, M.K., et al., *IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells.* J Immunol, 2001. **166**(9): p. 5530-9.
583. Gregori, S., et al., *Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway.* Blood, 2010. **116**(6): p. 935-44.
584. Harizi, H. and G. Norbert, *Inhibition of IL-6, TNF-alpha, and cyclooxygenase-2 protein expression by prostaglandin E2-induced IL-10 in bone marrow-derived dendritic cells.* Cell Immunol, 2004. **228**(2): p. 99-109.
585. Murray, H.W., *Accelerated control of visceral Leishmania donovani infection in interleukin-6-deficient mice.* Infect Immun, 2008. **76**(9): p. 4088-91.
586. Kurkjian, K.M., et al., *Multiplex analysis of circulating cytokines in the sera of patients with different clinical forms of visceral leishmaniasis.* Cytometry A, 2006. **69**(5): p. 353-8.
587. Ansari, N.A., S. Saluja, and P. Salotra, *Elevated levels of interferon-gamma, interleukin-10, and interleukin-6 during active disease in Indian kala azar.* Clin Immunol, 2006. **119**(3): p. 339-45.
588. Babaloo, Z., P.M. Kaye, and M.B. Eslami, *Interleukin-13 in Iranian patients with visceral leishmaniasis: relationship to other Th2 and Th1 cytokines.* Trans R Soc Trop Med Hyg, 2001. **95**(1): p. 85-8.
589. de Medeiros, I.M., A. Castelo, and R. Salomao, *Presence of circulating levels of interferon-gamma, interleukin-10 and tumor necrosis factor-alpha in patients with visceral leishmaniasis.* Rev Inst Med Trop Sao Paulo, 1998. **40**(1): p. 31-4.
590. Cillari, E., et al., *In vivo and in vitro cytokine profiles and mononuclear cell subsets in Sicilian patients with active visceral leishmaniasis.* Cytokine, 1995. **7**(7): p. 740-5.
591. Vouldoukis, I., et al., *Interleukin-10 and interleukin-4 inhibit intracellular killing of Leishmania infantum and Leishmania major by human macrophages by decreasing nitric oxide generation.* Eur J Immunol, 1997. **27**(4): p. 860-5.
592. Wu, J., et al., *IL-10 inhibits the synthesis of migration inhibitory factor and migration inhibitory factor-mediated macrophage activation.* J Immunol, 1993. **151**(8): p. 4325-32.
593. Murray, H.W., et al., *Determinants of response to interleukin-10 receptor blockade immunotherapy in experimental visceral leishmaniasis.* J Infect Dis, 2003. **188**(3): p. 458-64.
594. Pearson, R.D. and R.T. Steigbigel, *Phagocytosis and killing of the protozoan Leishmania donovani by human polymorphonuclear leukocytes.* J Immunol, 1981. **127**(4): p. 1438-43.
595. McFarlane, E., et al., *Neutrophils contribute to development of a protective immune response during onset of infection with Leishmania donovani.* Infect Immun, 2008. **76**(2): p. 532-41.

596. Radsak, M., et al., *Polymorphonuclear neutrophils as accessory cells for T-cell activation: major histocompatibility complex class II restricted antigen-dependent induction of T-cell proliferation.* Immunology, 2000. **101**(4): p. 521-30.
597. Gosselin, E.J., et al., *Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3.* J Immunol, 1993. **151**(3): p. 1482-90.
598. Windhagen, A., et al., *Human polymorphonuclear neutrophils express a B7-1-like molecule.* J Leukoc Biol, 1999. **66**(6): p. 945-52.
599. Tacchini-Cottier, F., et al., *An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with Leishmania major.* J Immunol, 2000. **165**(5): p. 2628-36.
600. Venuprasad, K., S. Chattopadhyay, and B. Saha, *CD28 signaling in neutrophil induces T-cell chemotactic factor(s) modulating T-cell response.* Hum Immunol, 2003. **64**(1): p. 38-43.
601. Dunay, I.R., A. Fuchs, and L.D. Sibley, *Inflammatory monocytes but not neutrophils are necessary to control infection with Toxoplasma gondii in mice.* Infect Immun, 2010. **78**(4): p. 1564-70.
602. Neuenhahn, M., et al., *CD8alpha+ dendritic cells are required for efficient entry of Listeria monocytogenes into the spleen.* Immunity, 2006. **25**(4): p. 619-30.
603. Scumpia, P.O., et al., *CD11c+ dendritic cells are required for survival in murine polymicrobial sepsis.* J Immunol, 2005. **175**(5): p. 3282-6.
604. Hammad, H., et al., *Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen.* J Exp Med, 2010. **207**(10): p. 2097-111.
605. Ahuja, S.S., et al., *Dendritic cell (DC)-based anti-infective strategies: DCs engineered to secrete IL-12 are a potent vaccine in a murine model of an intracellular infection.* J Immunol, 1999. **163**(7): p. 3890-7.
606. Ghosh, M., et al., *Dendritic cell-based immunotherapy combined with antimony-based chemotherapy cures established murine visceral leishmaniasis.* J Immunol, 2003. **170**(11): p. 5625-9.
607. Tamura, T., et al., *The IRF family transcription factors in immunity and oncogenesis.* Annu Rev Immunol, 2008. **26**: p. 535-84.
608. Aliberti, J., et al., *Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells.* Blood, 2003. **101**(1): p. 305-10.
609. Schiavoni, G., et al., *ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells.* J Exp Med, 2002. **196**(11): p. 1415-25.
610. Tsujimura, H., et al., *ICSBP/IRF-8 retrovirus transduction rescues dendritic cell development in vitro.* Blood, 2003. **101**(3): p. 961-9.
611. Tamura, T., et al., *IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity.* J Immunol, 2005. **174**(5): p. 2573-81.
612. Suzuki, S., et al., *Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development.* Proc Natl Acad Sci U S A, 2004. **101**(24): p. 8981-6.
613. Schiavoni, G., et al., *ICSBP is critically involved in the normal development and trafficking of Langerhans cells and dermal dendritic cells.* Blood, 2004. **103**(6): p. 2221-8.
614. Ichikawa, E., et al., *Defective development of splenic and epidermal CD4+ dendritic cells in mice deficient for IFN regulatory factor-2.* Proc Natl Acad Sci U S A, 2004. **101**(11): p. 3909-14.
615. Gabriele, L., et al., *IRF-1 deficiency skews the differentiation of dendritic cells toward plasmacytoid and tolerogenic features.* J Leukoc Biol, 2006. **80**(6): p. 1500-11.
616. Salkowski, C.A., et al., *IL-12 is dysregulated in macrophages from IRF-1 and IRF-2 knockout mice.* J Immunol, 1999. **163**(3): p. 1529-36.
617. Masumi, A., et al., *IRF-8/ICSBP and IRF-1 cooperatively stimulate mouse IL-12 promoter activity in macrophages.* FEBS Lett, 2002. **531**(2): p. 348-53.

618. Wang, I.M., et al., *An IFN-gamma-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages.* J Immunol, 2000. **165**(1): p. 271-9.
619. Liu, J., et al., *Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein.* J Biol Chem, 2004. **279**(53): p. 55609-17.
620. Gautier, G., et al., *A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells.* J Exp Med, 2005. **201**(9): p. 1435-46.
621. Honda, K., et al., *Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10872-7.
622. Hoebe, K., et al., *Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways.* Nat Immunol, 2003. **4**(12): p. 1223-9.
623. Montoya, M., et al., *Type I interferons produced by dendritic cells promote their phenotypic and functional activation.* Blood, 2002. **99**(9): p. 3263-71.
624. Marckmann, S., et al., *Interferon-beta up-regulates the expression of co-stimulatory molecules CD80, CD86 and CD40 on monocytes: significance for treatment of multiple sclerosis.* Clin Exp Immunol, 2004. **138**(3): p. 499-506.
625. Lee, S.J., et al., *Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274).* FEBS Lett, 2006. **580**(3): p. 755-62.
626. Zhao, Z., et al., *IFN regulatory factor-1 is required for the up-regulation of the CD40-NF-kappa B activator 1 axis during airway inflammation.* J Immunol, 2003. **170**(11): p. 5674-80.
627. Bauvois, B., et al., *Types I and II interferons upregulate the costimulatory CD80 molecule in monocytes via interferon regulatory factor-1.* Biochem Pharmacol, 2009. **78**(5): p. 514-22.
628. Lim, W., et al., *Regulation of B7.1 costimulatory molecule is mediated by the IFN regulatory factor-7 through the activation of JNK in lipopolysaccharide-stimulated human monocytic cells.* J Immunol, 2005. **175**(9): p. 5690-700.
629. Barnes, B.J., et al., *Global and distinct targets of IRF-5 and IRF-7 during innate response to viral infection.* J Biol Chem, 2004. **279**(43): p. 45194-207.
630. Takaoka, A., et al., *Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors.* Nature, 2005. **434**(7030): p. 243-9.
631. Negishi, H., et al., *Negative regulation of Toll-like-receptor signaling by IRF-4.* Proc Natl Acad Sci U S A, 2005. **102**(44): p. 15989-94.
632. Tsujimura, H., et al., *Toll-like receptor 9 signaling activates NF-kappaB through IFN regulatory factor-8/IFN consensus sequence binding protein in dendritic cells.* J Immunol, 2004. **172**(11): p. 6820-7.
633. Zhao, J., et al., *IRF-8/interferon (IFN) consensus sequence-binding protein is involved in Toll-like receptor (TLR) signaling and contributes to the cross-talk between TLR and IFN-gamma signaling pathways.* J Biol Chem, 2006. **281**(15): p. 10073-80.
634. Honda, K., et al., *Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction.* Nature, 2005. **434**(7036): p. 1035-40.
635. Kawai, T., et al., *Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6.* Nat Immunol, 2004. **5**(10): p. 1061-8.
636. Honda, K., et al., *Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling.* Proc Natl Acad Sci U S A, 2004. **101**(43): p. 15416-21.
637. Dietrich, N., et al., *Murine toll-like receptor 2 activation induces type I interferon responses from endolysosomal compartments.* PLoS One, 2010. **5**(4): p. e10250.
638. White, L.C., et al., *Regulation of LMP2 and TAP1 genes by IRF-1 explains the paucity of CD8+ T cells in IRF-1-/- mice.* Immunity, 1996. **5**(4): p. 365-76.

639. Penninger, J.M., et al., *The interferon regulatory transcription factor IRF-1 controls positive and negative selection of CD8+ thymocytes.* Immunity, 1997. **7**(2): p. 243-54.
640. Ogasawara, K., et al., *Requirement for IRF-1 in the microenvironment supporting development of natural killer cells.* Nature, 1998. **391**(6668): p. 700-3.
641. Taki, S., et al., *IFN regulatory factor-2 deficiency revealed a novel checkpoint critical for the generation of peripheral NK cells.* J Immunol, 2005. **174**(10): p. 6005-12.
642. Ohteki, T., et al., *The transcription factor interferon regulatory factor 1 (IRF-1) is important during the maturation of natural killer 1.1+ T cell receptor-alpha/beta+ (NK1+ T) cells, natural killer cells, and intestinal intraepithelial T cells.* J Exp Med, 1998. **187**(6): p. 967-72.
643. Toshchakov, V., et al., *TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages.* Nat Immunol, 2002. **3**(4): p. 392-8.
644. Barbalat, R., et al., *Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands.* Nat Immunol, 2009. **10**(11): p. 1200-7.
645. Marre, M.L., et al., *Human integrin alpha(3)beta(1) regulates TLR2 recognition of lipopeptides from endosomal compartments.* PLoS One, 2010. **5**(9): p. e12871.
646. Kim, Y.M., et al., *UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes.* Nature, 2008. **452**(7184): p. 234-8.
647. Latz, E., et al., *TLR9 signals after translocating from the ER to CpG DNA in the lysosome.* Nat Immunol, 2004. **5**(2): p. 190-8.
648. Kagan, J.C., et al., *TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta.* Nat Immunol, 2008. **9**(4): p. 361-8.
649. Petrasek, J., et al., *Type I Interferons Protect From Toll-Like Receptor 9-Associated Liver Injury and Regulate IL-1 Receptor Antagonist in Mice.* Gastroenterology, 2010.
650. Tam, M.A. and M.J. Wick, *MyD88 and interferon-alpha/beta are differentially required for dendritic cell maturation but dispensable for development of protective memory against Listeria.* Immunology, 2009. **128**(3): p. 429-38.
651. Ozato, K., et al., *TRIM family proteins and their emerging roles in innate immunity.* Nat Rev Immunol, 2008. **8**(11): p. 849-60.
652. Rescigno, M., et al., *Dendritic cell survival and maturation are regulated by different signaling pathways.* J Exp Med, 1998. **188**(11): p. 2175-80.
653. Pahl, H.L., *Activators and target genes of Rel/NF-kappaB transcription factors.* Oncogene, 1999. **18**(49): p. 6853-66.
654. Salkowski, C.A., et al., *Impaired IFN-gamma production in IFN regulatory factor-1 knockout mice during endotoxemia is secondary to a loss of both IL-12 and IL-12 receptor expression.* J Immunol, 2000. **165**(7): p. 3970-7.
655. Tailor, P., et al., *The feedback phase of type I interferon induction in dendritic cells requires interferon regulatory factor 8.* Immunity, 2007. **27**(2): p. 228-39.
656. Sgarbanti, M., et al., *IRF-7: new role in the regulation of genes involved in adaptive immunity.* Ann N Y Acad Sci, 2007. **1095**: p. 325-33.
657. Kong, H.J., et al., *Cutting edge: autoantigen Ro52 is an interferon inducible E3 ligase that ubiquitinates IRF-8 and enhances cytokine expression in macrophages.* J Immunol, 2007. **179**(1): p. 26-30.
658. Silva, S.R., et al., *Immunosuppressive components of Ascaris suum down-regulate expression of costimulatory molecules and function of antigen-presenting cells via an IL-10-mediated mechanism.* Eur J Immunol, 2006. **36**(12): p. 3227-37.
659. Qin, H., et al., *IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of suppressor of cytokine signaling-3.* J Immunol, 2006. **177**(11): p. 7761-71.
660. McBride, J.M., et al., *IL-10 alters DC function via modulation of cell surface molecules resulting in impaired T-cell responses.* Cell Immunol, 2002. **215**(2): p. 162-72.
661. Vezys, V., et al., *Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection.* J Exp Med, 2006. **203**(10): p. 2263-9.
662. Kemball, C.C., et al., *Late priming and variability of epitope-specific CD8+ T cell responses during a persistent virus infection.* J Immunol, 2005. **174**(12): p. 7950-60.

663. Stumhofer, J.S., et al., *A role for IL-27p28 as an antagonist of gp130-mediated signaling.* Nat Immunol, 2010. **11**(12): p. 1119-26.
664. Iyer, S.S., A.A. Ghaffari, and G. Cheng, *Lipopolysaccharide-Mediated IL-10 Transcriptional Regulation Requires Sequential Induction of Type I IFNs and IL-27 in Macrophages.* J Immunol, 2010. **185**(11): p. 6599-607.
665. Okuyama, M., et al., *A novel in vivo inducible dendritic cell ablation model in mice.* Biochem Biophys Res Commun, 2010. **397**(3): p. 559-63.
666. Do, Y., et al., *Targeting of LcrV virulence protein from Yersinia pestis to dendritic cells protects mice against pneumonic plague.* Eur J Immunol, 2010. **40**(10): p. 2791-6.
667. Tewari, K., et al., *Poly(I:C) is an effective adjuvant for antibody and multi-functional CD4+ T cell responses to Plasmodium falciparum circumsporozoite protein (CSP) and alphaDEC-CSP in non human primates.* Vaccine, 2010. **28**(45): p. 7256-66.