AN INVESTIGATION OF HOW DENDRITIC CELL MIGRATION IS MODULATED DURING *LEISHMANIA MEXICANA* INFECTION

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Abstract

The functional migration of immune cells during *Leishmania* infection is imperative for the activation of a protective immune response. In particular, migration of dendritic cells (DCs) towards the lymphatic vessel network and CCL19-producing lymph node is a key step in the induction of adaptive immunity. Consequently, manipulation of chemokines and chemokine receptors presents a potential mechanism by which parasites can evade protective host immunity. Therefore, the aim of this thesis was to investigate whether *Leishmania mexicana* can influence immune cell migration and identify the mechanisms underpinning this.

Using a combination of *in vitro* and *in vivo* studies, the key finding presented in this thesis is that *L. mexicana* modulates DC migration following infection. While uptake of *L. mexicana* by DCs is associated with reduced surface expression of CCR7, this does not entirely account for the failure of DC migration and therapeutic targeting of DCs does not enhance protective immunity. Crucially, it is through production of cysteine protease B that *L. mexicana* can suppress migration of infected and bystander DCs. Parasites lacking cysteine protease B are less able to prevent DC migration and the activity of this important virulence factor is shown to be associated with cleavage of CCL19. In further experiments, it is also demonstrated that effective DC migration following *L. mexicana* infection may also be influenced by interactions with neutrophils and their NETs and by an early source of interleukin-4/13 acting directly on DCs.

Primarily, these findings demonstrate how *L. mexicana* can utilise immunomodulatory virulence products, like cysteine protease B to manipulate the

host immune response. Understanding these mechanisms is invaluable for the development of new drug and vaccine targets against *L. mexicana*, but may also aid in the development of new treatments for similar chronic infections.

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Author's Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

Date:

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List of Abbreviations

°C	degree Celsius
AKP	alkaline phosphatase
ANOVA	analysis of variance
APC	antigen presenting cell
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CCR	chemokine receptor
CD	cluster of differentiation
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CHR	contact hypersensitivity reaction
CXCL	chemokine (C-X-C motif) ligand
DC	dendritic cell
dDC	dermal dendritic cell
dLN	draining lymph node
DNA	deoxyribonucleic acid
DT	diphtheria toxin
DTR	diphtheria toxin receptor
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-aided cell sorting
FcR	Fc receptor
FCS	foetal calf serum

GM-CSF	granulocyte macrophage-colony stimulating factor
gp63	glycoprotein 63
HBSS	Hanks balanced salt solution
hr	hour
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
IVIS	intravital imaging system
kg	kilogram
LC	Langerhans cell
LCF	*leishmania* chemotactic factor
LEC	lymphatic endothelial cells
LN	lymph node
LPG	lipophosphoglycan
LPS	lipopolysaccharides
LV	lymphatic vessel
MEM	modified eagles medium
mg	milligram
MHC	major histocompatibility complex
ml	millilitre
mM	millimolar
MPO	myeloperoxidase

MZ	marginal zone
NET	neutrophil extracellular trap
ng	nanogram
nm	nanometre
OVA	ovalbumin
PALS	periarteriolar lymphoid sheaths
PBS	phosphate buffered saline
PGE	prostaglandin E
PKDL	Post kala-azar dermal leishmaniasis
pNPP	p-Nitrophenyl Phosphate
PSG	promastigote secretory gel
RNA	ribonucleic acid
ROI	region of interest
SCID	severe combined immune deficiency
SEM	standard error of mean
SGS	salivary gland sonicate
SLA	soluble *leishmania* antigen
STWS	scots tap water substitute
TCR	T cell receptor
Tfh	Follicular helper T cell
Th	T helper cell
Treg	T regulatory cell
μg	microgram
μl	microlitre

μm	micrometer

Chapter 1. Introduction

1.1. Leishmaniasis: a neglected global health risk

Leishmaniasis poses a significant risk to global health, with over 310 million people in the poorest countries across the world at risk of *Leishmania* infection (WHO, 2013). Due to under-reporting and poor diagnostic approaches it is not possible to accurately determine the true global burden of Leishmaniasis (Geurin *et al,* 2002). However, recent estimates suggest that the countries with the highest incidence of cutaneous disease are spread across South America, Sub-Saharan African and the Middle East, totaling upwards of 1.2 million cases (WHO, 2012).

Transmitted by the bite of the female *Phlebotomus* and *Lutzomyia* sandflies, this protozoan parasite can cause a spectrum of clinical manifestation. Some species, such as *L. major* and *L. mexicana* can cause cutaneous lesion formation, others like *L. donovani* and *L. infantum,* can disseminate to the viscera and infection will result in systemic disease (Desjeux, 2004) (Table 1.1.). The adaptation of the species to different niches within the host, such as the skin or the liver and spleen, has developed over millions of years during the evolution of the parasite along side the host immune response.

***Leishmania* (*L*) species**	**Disease**	**Clinical presentation**
L. major	Cutaneous	– Individual lesion/nodule – Potential dissemination to the mucosa (MCL) – Atrophic scarring – Lyphoadenopathy
L. mexicana		
L. braziliensis		
L. amazonensis		
L. donovani	Visceral	– Hepatosplenomegaly – Fever – Weight loss – Potential development of Post-kala Azar dermal leishmaniasis (PKDL)
L. infantum		

Table 1. Commonly observed clinical presentations of predominant *Leishmania* species (WHO, 2012).

Thus, the outcome of disease is largely influenced by the ability of the host immune system to initiate a protective response. The way in which the host immune response responds to the parasite is infection site specific and so understanding the host and parasite-derived factors that influence anti-*Leishmania* immunity is essential for the development of novel drug and vaccine targets.

Crucially, most of the current treatment options for cutaneous and visceral leishmaniasis are costly and elicit a number of adverse effects (Desjeux, 2004). The most common drugs for intralesional treatment of cutaneous infection are the pentavalent antimonials, which can cause contraindications including pancreatitis and cardiac arythmia (Oliveira *et al*, 2011). Thus, with the advent of emerging resistance to a number of the most commonly used drugs, such as the pentavalent antimonials and Miltesophine (Croft *et al*, 2006), the development of more efficacious therapeutics and prophylactic drugs or vaccines is imperative.

The pursuit of an effective vaccination target for this disease is ongoing, however, as yet there has been limited success (reviewed in Kumar and Engwerda, 2014). In order to progress with drug and vaccine discovery we have to begin to understand the complexities of the host-pathogen relationship during *Leishmania* infection. While earlier studies on *Leishmania* were largely responsible for establishing the Th1/Th2 paradigm of resistance/susceptibility to intracellular infection (reviewed Alexander and Brombacher, 2012), more recent studies have demonstrated that the dynamics of the host immune response and host/parasite interplay is significantly more complex than previously envisioned with innate immune cells, antigen presenting cells and

parasite material all playing pivotal roles in determining disease outcome. Due to the complexity of the host immune response it is conceivable that any modulation of immune cell functionality could be the difference between resistance and susceptibility and development of progressive chronic disease.

1.2. The host immune response to *Leishmania*

1.2.1. The host immune response and subsequent outcome of infection

Over the decades, research in the field of *Leishmania* immunology has demonstrated that the development of disease is largely dependent on parasite strain and host genetic background (Shaw *et al,* 1995). In the clinic, it has been observed that host genetics can be linked to susceptibility (reviewed Handman *et al,* 2005) and in the research laboratory this is mirrored by the heterogeneity of disease outcomes in different mouse strains. Perhaps the most notable comparison can be drawn between BALB/c mice and C57BL/6 mice which, following infection with *Leishmania major* (*L. major*) generate very different immunological responses. BALB/c mice develop non-healing lesions and progressive disease, whereas C57BL/6 mice form small lesions that heal quickly (Alexander *et al,* 1985, reviewed Sacks and Noben-Trauth, 2002). In this case, resistance and susceptibility is associated with the production of T helper 1 (Th1) and T helper 2 (Th2) associated cytokines respectively (Heinzel *et al,* 1989; Locksley *et al,* 1991). Following infection, the clearance of parasites and promotion of a healing response is reliant on IL-12 driven IFN-γ production from Th1 cells. The production of IFN-γ and TNF from these cells has been shown to be important for the production of intermediate nitric oxide synthase (iNOS) production in macrophages (Wilhelm *et al,* 2001; Wei *et al,* 1995), which is required for

intracellular killing of *L. major* parasites (Bogdan *et al,* 1990). Conversely, a Th2 response is directed by IL-4/IL-13 (Erb *et al,* 1996), IL-10 (Groux *et al,* 1999) and TGF-β, cytokines that supress parasite killing and promote a non-healing phenotype. However, during infection with the New World parasite *L. mexicana*, susceptibility, depending on the strain of mouse or site of infection, is not necessarily linked with a Th2 type response, but rather the lack of a robust Th1 response (Rodriguez-Sosa *et al,* 2001; Rosas *et al,* 2005). In addition, it has also been established that the Th2 cytokines, IL-4 and IL-13 may not simply counter-regulate Th1 immunity, and IL-4 may also play a protective role in driving Th1 immunity to *L. major* (Biedermann *et al,* 2001; Ehrchen *et al,* 2010). Thus, the dogma of the Th1/Th2 balance determining resistance or susceptibility may not be as clear as once thought, and further work is required to explore the immunity to *Leishmania* (reviewed by Alexander and Brombacher, 2012).

As our understanding of the host immune response during *Leishmania spp.* infection expands, it is increasingly evident that the immunological mechanisms and disease outcome of infection varies widely across species. Whilst, *L. major* is the most widely studied infection model for cutaneous infection, cutaneous infection with *L. amazonensis* or *L. mexicana,* or visceral infection with *L. donovani,* will often have very different, if not contradictory responses to those observed in *L. major* infection (McMahon-Pratt and Alexander, 2004). Importantly, infection with *L. mexicana* will lead to persistent infection in most mouse strains, even in *L. major* resistant C57BL/6 mice. Thus, how the parasite can evade host clearance mechanisms and manipulate the host immune response to persist and cause chronic infection has been extensively questioned.

Classically, infection models have been used to investigate the immunology of disease, where parameters such as parasite-specific immunoglobulin production, T cell responses and the development of lesion size in mice have been used to chart the development of disease. Whilst such studies monitor parasite burden and associated immunity over 10-20 weeks (e.g. Arredondo and Perez, 1979; Jones *et al*, 2002), many reports have demonstrated that immune cell activation and migration during the first hours and days after infection or antigen exposure will be hugely influential in the development of a downstream adaptive response (Itano *et al*, 2003; Jenkins *et al,* 2001). Therefore, this classical approach may not be appropriate as it only examines the outcome of infection weeks after the initial stimulation of the immune response rather than investigating how the first hours of infection will be important in directing adaptive immunity.

1.2.2. Life cycle of *Leishmania*

In understanding the first immune interactions that mediate host susceptibility or resistance, it is first important to understand how disease is initiated. Various different species of sandfly can transmit *Leishmania* parasites, but the most common forms are *Phelobotomus papatasi* and *Lutzomyia longipalpis* - depending on geographical distribution (Killick-Kendrick *et al*, 1989; Sharma and Singh, 2010). *Leishmania* infection begins with the bite of infected female sandfly, which uses her mouthparts to tear the skin, forming a blood pool from which to feed. The sandfly will then regurgitate infective metacyclic promastigote parasites into the wound (Figure 1.1.), along with saliva and a plug of parasite secretory gel (PSG) (discussed in Section 1.2.3.).

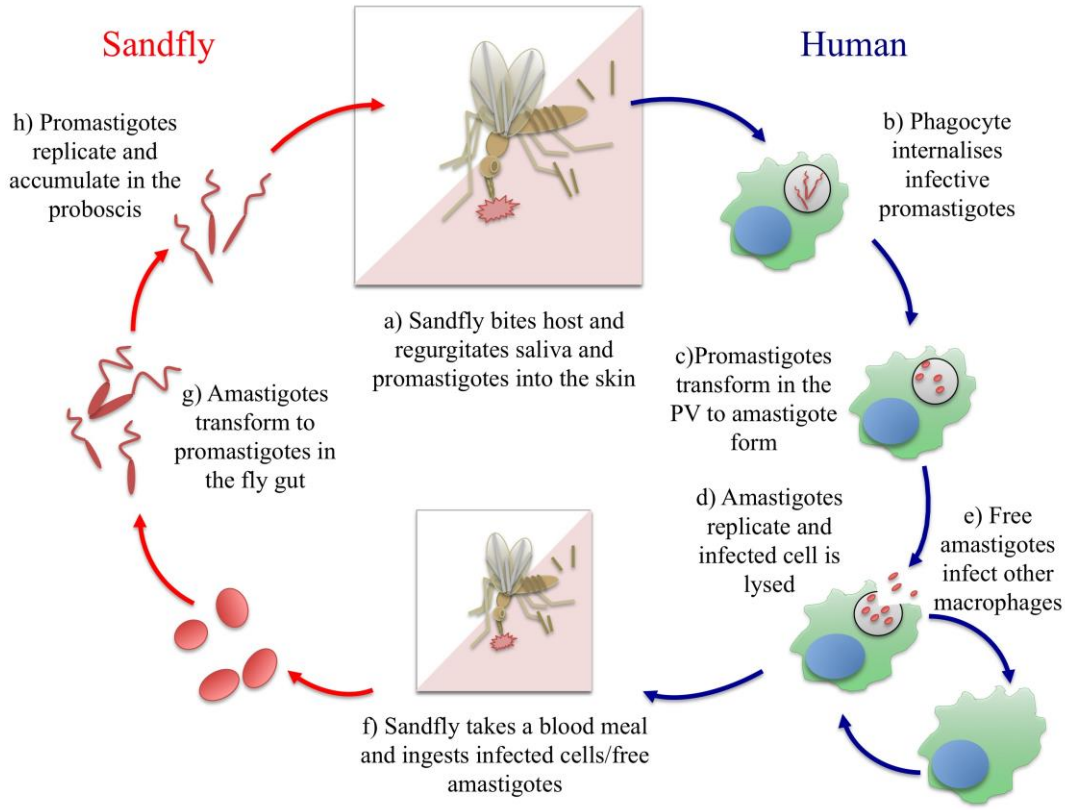


Figure 1.1. A schematic depicting the life cycle of the *Leishmania* parasite (CDC, 2014; Killick-Kendrick *et al*, 1989).

The metacyclic promastigote can be engulfed by a number of phagocytic immune cells, such as DCs and neutrophils (discussed below), although macrophages will act as the primary host cell for the parasite (Fig. 1.1b), where it can transform into the amastigote form (Fig. 1.1c), replicate and eventually cause lysis of the infected cell (Fig. 1.1d). The free amastigotes can rapidly infect other nearby macrophages (Fig. 1.1e), leading to the formation of the lesion. Eventually a sandfly may bite the host and ingest free amastigotes or amastigote-containing cells in the blood (Fig. 1.1f). The infected cells rupture in the gut of the fly and parasites transform into procyclic promastigotes (Fig. 1.1g) capable of replicating before migrating to the midgut and

proboscis where they mature into the infective metacyclic form ready to complete the cycle (Fig. 1.1h).

1.2.3. Initiation of the immune response to *Leishmania*

During the sandfly feeding process, the microvasculature of the skin is disrupted, triggering the production of inflammatory mediators, such as CXCL1 and IL-33 (described in Section 1.4.1.). The creation of this pro-inflammatory state provides the necessary environmental cues for innate cell recruitment and within hours of infection neutrophils will infiltrate the infected dermal tissue (von Stebut *et al* 2007; Tacchini-Cottier *et al*, 2000; Peters *et al,* 2008). These neutrophils rapidly develop an epidermal 'plug', as well as internalising parasites and triggering the recruitment of macrophages and DCs required for the generation of adaptive immunity - which will be discussed in more detail later.

1.2.4. Sandfly immunomodulatory factors

Importantly, it is not only host inflammatory mediators that are at play during this initial stage of infection. Sandfly saliva has also been proven to directly affect disease outcome (Titus and Ribeiro, 1988). Titus and Ribeiro (1988) first examined the effects of sandfly saliva on the development of disease following infection with *L. major*. The group demonstrated that co-inoculation with salivary gland lysate of *L. longipalpis* and a high dose of *L. major* promastigotes (10^5) resulted in enhanced lesion growth in both resistant (CBA) and susceptible (BALB/c) mice and enhanced parasite burden. Following this work, a study by Belkaid *et al* (1998) endeavoured to investigate the effects of sandfly saliva in a more naturalistic infection model i.e. a low infective dose of *L. major* promastigotes *major* (10^3) and salivary gland sonicate

(SGS) from *P. papatasii.* The study demonstrated that SGS promotes a non-healing response, enhances parasites burden and stimulates the generation of Th2 type immunity, and that this is attributable to enhanced cell recruitment and subsequently the provision of a host cell for parasite replication (Belkaid *et al,* 1998).

In addition, sandfly saliva has been shown to contain a number of parasite associated immunomodulatory compounds. As mentioned above, prior to feeding the sandfly must first regurgitate a "plug" of parasite-derived secretory gel (PSG) along with metacyclic promastigotes (Bates *et al,* 2007). It was in 2002, that Rogers *et al* first identified PSG and demonstrated that infection was significantly exacerbated by co-injection of purified PSG following a low initiating dose of *L. mexicana* parasites (10^3 promastigotes). Rogers *et al* (2004) then went on to characterise the main exacerbatory component of PSG, as filamentous proteophosphoglcans (fPPG), and more recently, has described how this substance can encourage macrophage recruitment to the skin and stimulate arginase activity in the definitive host cell, aiding parasite survival and replication in the macrophage (Rogers *et al,* 2009). Taken together, these studies demonstrate that fPPG, containing PSG can manipulate macrophage recruitment, activation and polyamine production and in this way can enhance parasite burdens and exacerbate disease.

Furthermore, when the parasite is deposited in the host skin promastigotes and amastigotes begin to produce various secretory products, such as Leishmania chemotactic factor (LCF) and cysteine proteases, which can influence host cell recruitment, migration and function. These factors and their effects will be discussed in more detail in Section 1.6.

1.2.5. The kinetics of immune cell recruitment following infection

Chemokines and cytokines orchestrate the recruitment and migration of immune cells during steady state and in infection. The tightly regulated expression of chemokines and chemokine receptors ensure a coordinated recruitment of immune cells following infection and allow for the constant surveillance for potentially harmful antigens (Esche *et al,* 2005). The kinetics of immune cell migration following *Leishmania* infection can directly influence antigen presentation and the subsequent generation of an effector T cell response (Muller *et al*, 2001; Ato *et al,* 2002).

As mentioned above, neutrophils are among the first cells to arrive at the site of infection, recruited by various host and parasite inflammatory mediators (i.e. CXCL1, LCF, PSG). Neutrophils are key for the production of chemokines that will recruit macrophages and DCs (Figure 1.2) such as CCL3 which rapidly recruits macrophages and DCs to the site of infection. As early as 24 hours post infection, macrophages and DCs have been identified in infected tissue (van Zandbergen *et al*, 2004).

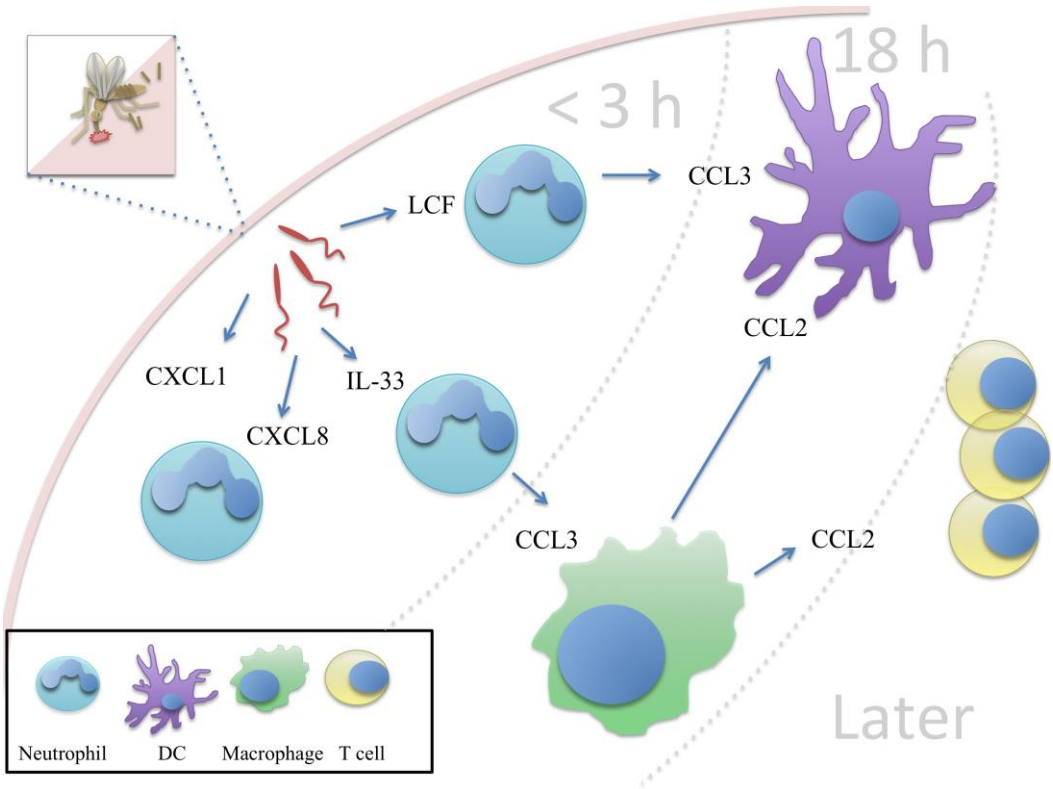


Figure 1.2. Diagram depicting cell recruitment at the site of infection and approximate times following infection that different cell types infiltrate tissue.

Leishmania infection also directly induces production of CCL2 (also known as monocyte chemoattractant protein-1β (MCP-1β)), from proximal endothelial and smooth muscle cells at the bite site. CCL2 is able to attract a variety of $CCR2^{+}$ cells, such as DCs, monocytes, NK cells and T cells (Daly and Rollins, 2003) and in turn activated monocyte/macrophage produce more CCL2 (Arias *et al*, 2007), further increasing recruitment of migratory immune cells, particularly DCs.

The complex relationships between neutrophils, macrophages, subsets of DCs and the parasite makes it challenging to fully understand the key roles each cell type plays in the acquisition of parasite antigen and the subsequent initiation of adaptive

immunity. Of particular importance is the capacity of DCs to present parasite-derived material in the draining lymph node, as this is a key event in the activation of naïve T $CD4^+$ cells (Itano *et al,* 2003) and also represents a potential route for parasite immune-evasion (de Souza Leao *et al,* 1995).

1.3. DCs and Leishmania

1.3.1. DC subsets present in the skin and infection site

DCs are very effective antigen presenting cells that are capable of activating naive T cells, via expression of co-stimulatory molecules and presentation of antigen in the context of MHC Class II-TCR interactions. In addition, DCs also have a pivotal role in T cell differentiation and polarisation of the cellular and humoral immune responses (Jenkins *et al,* 2001; reviewed Itano *et al,* 2003). The distribution of these migratory cells throughout the host tissues allows the DC to act as sentinel cells for pathogens (Moseman and Williman, 2004). However, the kinetics and extent of capture and presentation of *Leishmania* parasites and of parasite-derived antigens is yet to be fully defined. Local dendritic cells, resident at the site of infection, may be important for successful antigen presentation and activation of a T cell response and subsequently migrate to lymph node (Itano *et al,* 2003; Ng *et al,* 2008;Von Stebut *et al,* 1998). However, the skin hosts a number of distinct dendritic cells subsets, each with a potential role in processing, trafficking or presenting antigen. It has been shown that DC subsets received by the draining lymph nodes are primarily tissue derived DCs, such as Langerhans cells (LCs) and dermal DCs (dDCs), subsets that reside primarily in the skin and monocyte derived DCs (moDCs) that are derived

from monocytes and are rapidly recruited to the site of inflammation (reviewed by Itano *et al*, 2003; Serbina *et al,* 2003, Sen *et al,* 2010).

1.3.2. DC interaction with *Leishmania* promastigotes

Since their characterisation in the 1970s, a number of skin sensitisation studies demonstrated that MHC Class II-expressing LCs, were instrumental in the proliferation of antigen-specific T cell proliferation (Stingl *et al,* 1978). Thus, LCs were long thought of as the critical cells in the skin for antigen transport to the draining lymph node and T cell priming (Moll *et al*, 1993; Moll *et al,* 1998). However, it has now been suggested that specific "danger signals" in the epidermis are required for the upregulation of MHC Class II and mobilisation of these cells to the LN (Matzinger *et al,* 1994; Enk and Katz *et al,* 1992; reviewed by Romani *et al,* 2012).

To study these events *in vivo*, Ng *et al* (2008) used intravital multiphoton microscopy to visualise the movement of dDCs and Langerhans cells in the skin. They observed how both cell types "patrol" the skin for foreign antigens during steady-state and *L. major* infection. The authors demonstrated how Langerhans cell are less motile than dDC, using long processes to survey the local environment, where as dDCs migrate to the site of tissue damage within minutes, arresting at the site of infection and efficiently phagocytosing promastigotes. Interestingly, this reduction in motility of dDCs was observed in both parasitised and non-parasitised cells, suggesting that there must be an environmental factor governing chemotaxis of the dDCs in the local area of inflammation.

In light of these findings and with the creation of transgenic mice, it has become increasingly evident that that the transport of parasite antigen is not reliant on LCs alone. A study by Bennett *et al* (2005) investigated this by depleting the LC population using diphtheria toxin to induce depletion in diphtheria toxin receptor-Langerin mice to show that LCs are not essential for the development of a contact hypersensitivity reaction. Localised cutaneous swelling occured in the affected area in the absence of LC, suggesting that other skin DCs, such as dDCs will have a more prominent role in the development of T cell immunity.

Similarly, Ritter *et al* (2004) have demonstrated that LCs are not essential for the development of a $CD4^+$ T cell response during *L. major* infection. Instead, the authors demonstrated that *L. major* parasitised $Langerin^-$ dDCs in the skin will efficiently migrate to the paracortex of the draining lymph node over the course of 3 days post-infection. Further to this, the authors showed that *ex vivo* stimulation of T cells liberated from the same draining lymph node were specific for *L. major* antigen. In fact, Kautz-Neu *et al* (2011) established that LCs might even promote the expansion of regulatory T cells (Tregs). Whereby, C57BL/6 mice selectively depleted of $Langerin^+$ LCs (using the DTR/ DT system) developed less severe lesions before healing, and reduced numbers of Tregs were detected in the lesions.

Together these studies suggest that the migration of skin resident DCs, in particular dDCs, is key for the presentation of antigen to, and proliferation of, antigen specific-T cells, at least in the context of *L. major* infection. Whilst, these studies suggest that LCs do not appear to migrate from the skin, these cells may produce important migratory cues for other dendritic cell subsets and/ or macrophages. Some evidence suggests that *L. major* will invade and activate foetal skin derived DCs, which are

described as “LC-like DCs”, resulting in the production of IL-12 (von Stebut et al, 1998). IL-12 is important in promoting the development of a Th1 response in *Leishmania* infection, and so it is possible that LCs might contribute to Th1 polarisation from the lesion directly. Alternatively, as Kautz-Neu *et al* (2011) suggest, these cells may also influence the polarisation of T cells to a Treg phenotype and in this way may cause exacerbation of disease. Thus, the controversial role of LCs during *Leishmania* infection remains to be fully defined.

Similarly, the recruitment of circulatory monocytes and DCs to the site of *L. major* infection is thought to be largely regulated by the expression of neutrophil derived CCL3 (Charmoy *et al,* 2010). Leon *et al* (2007) demonstrated that monocytes are recruited into the infected skin and subsequently differentiate into moDCs. It has been reported that these cells are an important source of iNOS in *L. major* infection (Serbine *et al,* 2003) and have the capacity to rapidly migrate, as such they could have an accessory role in antigen trafficking to the draining lymph node and production of IL-12 in the lymph node. Interestingly, Petritus *et al* (2012) recently demonstrated that the recruitment of moDCs was impaired during *L. mexicana* infection, when compared with *L. major* infection. The authors suggested that recruitment into infected skin and possibly migration away from the site of infection, to the draining LN, was comparatively reduced in *L. mexicana* infection. However, this study does not take into consideration whether the reported reduction in moDC recruitment will have a downstream effect on the population of moDCs that are available for migration to the draining LN. Therefore, from this evidence it is not possible to determine if migratory capacity of moDCs to the draining LN is impaired during *L. mexicana* infection or whether the reduction in the number of moDCs

reaching the LN is because fewer moDCs are present in the infected tissue. Furthermore, this study does not investigate the role of these moDCs in trafficking parasite antigen or stimulating T cell activation and thus it is not clear what influence these cells have on the adaptive immune response to *Leishmania* antigen.

1.3.3. *Leishmania mexicana* can manipulate DC functionality

It has been discussed that infection with *L. major* and *L. mexicana* infection results in very different disease phenotypes, but what are the reasons behind this disparity? Both species have been reported to have a whole host of virulence mechanisms for manipulation of the host cell function (reviewed Bogdan and Rollinghof, 1998). However, one key virulence mechanism that *L. mexicana* possesses is the production of cysteine proteases, specifically cysteine protease B - which will be discussed in more detail below (Mottram *et al,* 1996; Alexander *et al,* 1998). Indeed, it has been demonstrated that the parasite can modulate essential intracellular signaling, expression of surface molecules and production of cytokines required for successful presentation of antigen to and activation of $CD4^+$ T cells (reviewed Mottram *et al* 2004). Cameron *et al* (2004) and recent work by Shweash *et al* (2011), have shown that macrophage production of IL-12 is inhibited by *L. mexicana* promastigotes, in part through degradation of NFκB signaling and the subsequent modulation of PGE_2, iNOS and arginase activity. More recently, Contreras *et al* (2014) examined how the parasite can affect DC function *in vitro*. The group demonstrated that IL-12 production (specifically IL-12p70) was inhibited following exposure to *L. mexicana* and that expression of MHC Class II and the co-stimulatory molecules, CD80 and CD86 were all reduced. Further to this, when pulsed with exogenous antigens, DCs exposed to *L. mexicana* failed to stimulate antigen-specific T cells to produce high

levels of IFN-γ *in vitro*. These studies suggest that *L. mexicana* can suppress the presentation of antigen and production of key cytokines during infection and thus can inhibit the generation of Th1 immunity.

1.3.4. DC migration to the lymphatic vessels and the draining LN

In order to activate naïve $CD4^+$ T cells, DCs must first become activated in the periphery and migrate through the lymphatic vessels to the draining lymph node. During inflammatory conditions, the DC population in the LN is largely made up of lymph-derived DCs (Pugh *et al,* 1983). It has been demonstrated that even in the presence of soluble antigen, which will passively drain to the LN during infection, poor migration of lymph derived DCs to the draining LN will correlate with a reduced adaptive immune response (Itano *et al,* 2003). However, the exact mechanism of how DCs migrate from tissue into the lymphatic vessels LV remains ill defined. It has been demonstrated that DCs will migrate through channels formed by interstitial fluid flow (Schmid-Schonbein, 1990) and when the cells reach the vessels the DCs enter the initial lymphatic capillaries through gaps in the basement membrane (Pflicke and Sixt, 2009). These preformed portals allow for integrin-independent squeezing of DCs into the vessels (Lammerman *et al,* 2008).

It has also been shown that DCs require the expression of a number of chemokine receptors during migration in steady-state and in inflammatory conditions. Specifically, studies have demonstrated that functional migration of DCs and macrophages to the draining lymph node is reliant on the expression of CCR7 (Ohl et al, 2004; Forster *et al,* 1999). As such, CCR7-deficient mice have profound impairment of LN biogenesis and adaptive immunity. CCR7 deficient mice have

abrogated T cell and B cell homing to the LN, LN structural defects, and impaired T cell and humoral immune responses (Forster *et al,* 1999). Ohl *et al* (2004) further demonstrated that CCR7-deficient mice have significantly impaired migration of dermal and epidermal DCs to the skin draining LN and that this impairs activation of a T cell response.

Extensive studies of DC migration in lymphatic vessels (LVs) and draining lymph nodes (LN) have confirmed the importance of CCR7 sensing of CCL19 and CCL21 (Jang *et al,* 2006; Robbiani *et al*, 2000*;* Schumman *et al*, 2010; Lammerman *et al*, 2008). CCL19 is primarily produced by stromal cells (Ngo *et al*, 1999) and other DCs, where as CCL21 is expressed in two forms - CCL21-Leu, which is produced by lymphatic endothelial cells that form lymphatic capillaries, and CCL21-Ser, expressed by high endothelial vessels (HEV) in the secondary lymphatic tissues (Gunn *et al,* 1998). Tal *et al* (2011) have utilised *in vivo* imaging techniques, to visualise small concentrated regions of immobilised CCL21 on the basal membrane of the vessel. The authors demonstrated that the interaction between CCR7-expressing DCs and the immobilised CCL21 facilitates entry of the DC into the vessel as in $CCR7^{-/-}$ mice, DCs fail to adhere to the basal membrane and do not transmigrate into LVs. Further work carried out by Sixt and colleagues showed that bone-marrow derived DCs (bmDCs) rapidly migrate towards immobilised CCL21 *in vitro* and that the presence of DCs causes release of a soluble, cleaved version of CCL21, which can attract more CCR7-expressing DCs (Schumann et al, 2010). In addition, studies using *plt/plt* mice, that are naturally CCL19 and CCL21-Ser deficient, have demonstrated that both these chemokines are essential for development of the T cell zone and the migration of DCs to this area of the lymph

node and subsequent development of T cell immunity (Gunn *et al,* 1999; Forster *et al,* 1999).

However, whilst the recent development of intravital imaging techniques have gone a long way to elucidating some of the mechanisms required for DC migration towards the lymph node and throughout these tissues, the minutia of these mechanisms, and certainly how parasites such as *Leishmania* can manipulate this process, have yet to be defined.

1.3.5. DC migration during *Leishmania* infection

There is a body of evidence to suggest that *Leishmania* can modulate the expression of chemokine and chemokine receptors on the surface of DCs and that this will affect the recruitment into and migration out of the site of infection to the draining LV and LN. As has been discussed above, the migration of DCs to the LN is largely dependent on the CCR7/CCL19 and CCL21 migration axis. A study by Ato *et al* (2006) used *plt/plt* mice to investigate the importance of CCL19 and CCL21-Ser during *L. donovani* infection. The authors demonstrated that the mobilisation of DCs from the marginal zone (MZ) to the periarteriolar lymphoid sheath (PALs) region of the spleen was essential for the generation of protective immunity and control of parasite burden. However, it should also be considered that *L. donovani* parasites primarily infect cells in the spleen, and so it is possible that the altered architecture/ change in cell populations in spleen of *plt/plt* mice, may have an inherent influence on the outcome of infection (Mori *et al,* 2001). However, in addition, Ato *et al* (2002) also present convincing data to suggest that the chronicity of the infection is associated with a downregulation of CCR7 on the surface of DCs (Ato et al, 2002), mediated by enhanced IL-10 production. These studies, coupled with evidence to

suggest that there is a significant decrease in CCL21- and CCL19-expressing $gp38^{+}$ stromal cells in the T cell zones of the spleen during *L. donovani* infection (Ricart et al, 2011; Shummann, 2010), suggest that diminished DC activation and altered LN chemokine expression cause a dysregulation in DC migration that may contribute to a prolonged, non-healing infection in visceral leishmaniasis.

It has been demonstrated that *L. major* parasites produce virulence factors -which will be discussed further below – which can influence DC migration. Ponte-Sucre *et al* (2001) have demonstrated that *L. major* promastigote derived LPG can actively inhibit LC emigration from ear skin explants. In support of this, Jebbari *et al* (2002) suggested that *L. major* promastigotes can produce factors (possibly LPG) that inhibit DC migration *in vitro.*

Furthermore, Sato *et al* (2000) have shown that *L. major* infection is exacerbated in CCR2-deficient mice (Sato *et al,* 2000), and this can be attributed in part to the diminished ability of DCs to relocate to the draining lymph node (Steigerwald and Moll, 2005). A recent study by Jimenez *et al* (2010) has shown that during *L. major* infection CCR2 is essential for migration of LCs to the draining lymph node, and triggers the expression of co-stimulatory factors. With the aid of $CCR2^{-/-}$ mice, this group showed that CCR2 expression was required for optimal production of CCL19 and that dysregulation of CCL19 impairs LC migration. Additionally, Jimenez *et al* (2010) have shown that CCR2 activates the NFκB signalling pathway, essential for production of IL-12p70. Therefore, an apparent lack of LC migration, coupled with reduced expression of co-stimulatory factors and IL-12p70 may result in fewer encounters with naïve T cells and a reduced capacity to drive T cell activation and differentiation.

Interestingly, Iezzi *et al* (2006), have suggested that migration of skin derived DCs, such as dDCs, arriving from the lymph to the LN is not essential for early presentation of Ag to T cells, but rather that lymph node resident DCs are the main antigen presenting population following infection. As discussed previously, Itano *et al* (2003), have demonstrated that lymph derived DCs are essential for the generation of a strong T cell immune response, and so whilst this study suggests that soluble antigen will have a role in the development of T cell immunity it is likely that lymph derived DCs will also have an important influence in this process. However, to date a working model of how soluble versus particulate antigen influences the T cell response during *Leishmania* infection has not been clarified.

With this in mind, it is important to take into consideration how needle infection carried out in the laboratory may differ from sandfly mediated infection and how parasite/parasite material inoculation may affect the presentation of antigen and generation of adaptive immunity. Needle injection will typically comprise of a high dose of promastigotes (10^5-10^7) with a small amount of dead parasite material, whereas during natural sandfly mediated infection few viable/ infective parasites are deposited in the skin with other disease exacerbatory factors such as sandfly saliva, PSG, fPPG (Titus and Ribeiro *et al,* 1988; Rogers *et al,* 2002; Rogers *et al,* 2004). Therefore, how these differences might direct the immune response must be taken into consideration when designing new drugs or vaccines for the disease.

Importantly, as has been discussed above, during chronic *L. mexicana* infection in C57BL/6 mice, evidence suggests that moDC migration is modulated relative to *L. major* infection, with fewer monocytes recruited to the site of infection, less iNOS expressed by the cells that are recruited and reduced accumulation of moDCs in the

draining LN after infection (Petritus *et al,* 2012). However, from this study it is not clear whether the reduction in the population of moDCs in the LN is due to parasite modulation of moDC migration or a downstream effect from the reduction in total number of moDCs at the infection site. Therefore, it is not known whether the migratory capacity of DCs towards the LV and LN during *L. mexicana* infection is modulated by the parasite. Equally, if this is the case, the mechanism by which the parasite can exert this effect is not understood, and any possible role for parasite virulence products has not yet been identified. Further to this, it should be noted that the kinetics of cell recruitment and migration during *L. mexicana* infection is complex and the role of other immune cells, such as neutrophils and associated innate immune mechanisms, like NET formation, might have important influence on DC migration. However, to date the functions of these cells, and cell products, during *L. mexicana* infection remains ill defined.

1.4. Neutrophils and *Leishmania*

1.4.1. Initial neutrophil recruitment following *Leishmania* infection

Numerous studies have demonstrated that following infection, whether via sandfly bite or needle inoculation, neutrophils mobilise rapidly and infiltrate the infected tissue (Peters *et al,* 2008; Hurrell *et al,* 2015; Tachini Cottier *et al,* 2000; Beil *et al,* 1992). As discussed above, this inflammatory response is largely regulated by chemokine expression at the infection site and epidermal cell expression of CXCL1 (an efficient neutrophil chemoattractant) peaks at 8 hours post-infection, recruiting the initial wave of neutrophils to the damaged tissue (Muller *et al*, 2001; Vester *et al*, 1999). In addition to chemokine expression, it has been shown that skin damage will

trigger alarmin release (Bianchi *et al*, 2006*)*. For example, IL-33 is more commonly implicated in autoimmune conditions, such as psoriasis, but this cytokine has been shown to contribute to the rapidity of infiltration of neutrophils during *Leishmania* infection (Heuber *et al*, 2011; Kaye and Scott, 2011). Therefore, to begin to understand the complex role of neutrophils in the development of an adaptive immune response, Tacchini-Cottier *et al* (2000), used monoclonal antibody (NIMP-R14) to deplete neutrophils during the first weeks of *L. major* infection. The authors demonstrated that NIMP-R14 treatment of BALB/c mice results in exacerbation of disease, impaired parasite intracellular killing, and promotion of a non-healing Th2 type response (Tacchini-Cottier *et al,* 2000). In contrast, Hurrell *et al* (2015) have recently demonstrated that following *L. mexicana* infection, C57BL/6 mice transiently depleted of neutrophils (1A8 mediated) - during the first 72 hours of infection - or neutropaenic *Genista* mice, will successfully resolve ear dermis lesions 8 weeks later. Therefore, as even transient neutropaenia has such a significant impact on the outcome of disease in both *L. major* and *L. mexicana* infection (albeit distinctly species specific), it is evident that neutrophils play an essential role in directing the T helper response, however it remains unclear what role(s) that may be.

As described above, the *Leishmania* parasite and the sandfly can impact on the recruitment of these inflammatory cells. Curiously, it was also discovered that after the initial peak of neutrophil infiltration (approximately 3 days post-infection) (Peters *et al,* 2009; Thalhofer *et al,* (2011), a secondary wave of neutrophil recruitment occurred following *L. major* infection (Peters *et al,* 2009). Peters *et al* (2009) demonstrated that 7 days after infection a distinct surge in neutrophils was detected in C57BL/6 mice. It could be the case that latter wave of neutrophil

migration is important for the formation of lesion granuloma, and thus the initial wave and secondary wave of neutrophil recruitment may serve distinct purposes for the development of infection (Thalhofer *et al,* 20011). Equally this latter stage of neutrophil infiltration could as easily have implications for chemokine expression and modulation of DC/macrophage recruitment and/or parasite dissemination later in infection.

1.4.2. Neutrophil recruitment of other immune cell types

Aside from orchestrating the migration of macrophages, dendritic cells and T cells after infection, evidence suggests that neutrophils can directly engulf promastigotes. Electron microscopy of *L. major* infected footpads show parasitised neutrophils as early as 6 hours after infection (Lima *et al*, 1998), however, as yet the fate of the parasite once inside the neutrophil remains unclear.

Laskay *et al* (2008) have suggested that neutrophils provide a "hiding place" for *L. major* parasites, allowing them to evade FcR-mediated uptake by macrophages, and thus avoid intracellular killing. This has become known as the Trojan horse hypothesis. Whilst inside the neutrophil, the parasite has been shown to manipulate the biochemistry of the cell, influencing the production of cytokines and chemokines *in vivo* (Laskay *et al,* 2008) as well as inducing neutrophil apoptosis (Ribeiro-Gomes *et al,* 2004; Ribeiro-Gomes *et al,* 2012; Laskay *et al,* 2008, Peters *et al,* 2008). Laskay *et al* (2008) provide evidence to show that these parasitised neutrophils upregulate production of CCL4 triggering macrophage recruitment and phagocytosis of the infected neutrophil (Laskay *et al,* 2008). However, macrophages interacting with apoptotic, *Leishmania*-containing neutrophils have been associated with an

increase in parasite burden that is dependent on TGF-β1 and PGE_2 production (Ribeiro-Gomes *et al,* 2004), further supporting the hypothesis that parasite entry into neutrophils allows for quiescent access into the macrophage.

Conversely, advances in imaging techniques have prompted conflicting views of the role of neutrophil as a "Trojan horse" cell. Notably, Peters *et al* (2008) performed *in vivo* imaging of neutrophil migration in the *L. major* infected ear dermis after experimental infection by sandfly bite. This work demonstrated that neutrophils phagocytose a large proportion of free parasites, and in this way can act as a primary host cell. Whilst the authors show that parasites within neutrophils remain viable and infective, they did not observe macrophage-mediated phagocytosis of parasitised neutrophils, as was previously hypothesised by Laskay *et al* (2008). Instead, it was demonstrated that macrophages directly phagocytose parasites and thus, it was suggested that neutrophils provide an essential niche for parasite maturation and have a role in "rescuing" parasites from extracellular killing. Supporting this, Ribeiro-Gomes et al (2012), suggested that parasite-containing neutrophils are instead captured by dDCs and are preferentially phagocytosed to non-parasitised neutrophils. In addition, the majority of parasite-containing dDCs in the skin also stain for neutrophil-associated markers of apoptosis, suggesting that neutrophils are ingested by the DCs (Ribeiro-Gomes *et a*l, 2012). Interestingly dDCs that have engulfed parasitised neutrophils also show reduced expression of activation markers, compared with uninfected dDCs. Together these data suggest that parasitised neutrophils are capable of hijacking a large proportion of skin-resident dDCs, resulting in a supressed DC phenotype with a reduced capacity to prime naïve T cell responses.

Overall these studies suggest an essential role for neutrophils as an early host cell for *Leishmania*, allowing for the propagation of the parasite, transfer to other phagocytic cells and passage of parasites through neutrophils could be detrimental to the host.

1.4.3. Neutrophils recruit DCs to the site of infection

It is largely unknown how neutrophils will interact with DC during *Leishmania* infection. A number of studies have shown that the early accumulation of neutrophils in the infected skin enables subsequent recruitment of DCs, by providing an essential source of chemokines that will direct cellular kinetics. In the case of *L. major* infection, there is evidence to suggest that neutrophil derived neutrophil-derived CCL3 is essential for DC recruitment in the first days of *L. major* infection (Charmoy *et al,* 2010). Charmoy *et al* (2010) examined resident and recruited DCs in the *L. major*-infected ear dermis in both resistant and susceptible mouse strains. Resistant C57BL/6 mice show higher levels of CCL3, with more Langerhans cells (LC), monocyte-derived DCs and dermal DCs than are found in susceptible BALB/c mice. Further to this, depletion of neutrophils (using the NIMP-R14 antibody) or blocking CCL3 (pharmacologically or using $CCL3^{-/-}$ animals) significantly reduced the number of DCs found in the ear dermis in both mouse strains. Together, these data provide evidence that neutrophil-derived CCL3 plays an essential role as a dendritic cell chemoattractant and that resistance to the parasite in the C57BL/6 background may be due to high levels of CCL3 production (Charmoy *et al,* 2010).

However, to date the mechanism of how neutrophils interact with and influence the behaviour of DCs at the site of infection remains ill defined. As has been discussed previously, Ribeiro-Gomes *et al* (2012) have provided evidence to support a scenario where neutrophils first engulf promastigotes and then DCs will engulf these

apoptotic parasite-containing neutrophils. In this way it has been suggested that neutrophils will influence the function of the DCs. However, conversely Ng *et al* (2008) have demonstrated that dDCs are capable of phagocytosing *L. major* parasites directly and do not require this neutrophil engulfment step.

Interestingly, Hurrell *et al* (2015) have recently used 2-photon imaging to quantify DC recruitment to the site of *L. mexicana* infection in the ear dermis. This study suggests that neutrophils may inhibit DC recruitment and thus engulfment of parasites directly following infection. Thus, this phenomenon may affect parasite antigen presentation and development of a T helper response. In turn, this may explain the enhanced disease resolution experienced in neutropaenic mice following *L. mexicana* infection. Thus, it is important to determine the functions of these cells and their ability to regulate DC function and affect the development of adaptive immunity.

1.4.4. The function of NETs in *Leishmania* infection

The rapid uptake of parasites and recruitment of further leukocytes are not the only functions of neutrophils during the initial stages of infection. Under certain stimuli, neutrophils can extrude their nuclear material, forming large swathes of filamentous chromatin and histones known as neutrophil extracellular traps (NETs) (Brinkman, 2007). Guimares-Costa *et al* (2009) have shown that Lipophosphoglycan (LPG) on the surface of *L. amazonensis* promastigotes can trigger the formation of NETs, which incorporate leishmaniacidal histone arrangements and, as such, are very effective at parasite killing *in vitro*. Whilst, these experiments are intriguing, it

should be considered that although NETs may kill a large proportion of promastigotes *in vitro*, it is possible that some viable parasites will survive NET mediated killing *in vivo.* Indeed, more recent work has suggested that *L. infantum* is able to escape NET-mediated killing through expression of 3'-nucleotidease/nuclease (Guimaraes-Costa *et al*, 2014). Similarly, *L. donovani* may resist the anti-microbial activity of NETs through expression of LPG (Gabriel *et al*, 2010).

Recently, other research groups have used a reductionist approach to study the role of NETs in both infectious and autoimmune disease (Li *et al,* 2010; Hemmers *et al*, 2011; Rohrbach *et al,* 2012). Peptidylarginine deiminase 4 (PAD4) is essential for the citrullination of histones (Wang *et al,* 2009) and therefore required for the generation of NETs. Indeed, PAD4-deficient mice are unable to form NETs during bacterial infection (Li *et al* 2010), which results in impaired killing of bacteria and a subsequent increase in acute disease. However, in a recent study by Rohrbach *et al* (2012), it is suggested that NETs may not be essential in development of chronic inflammation, at least when using an autoantibody-mediated model of arthritis. However, the influence of NETs on other cells, such as DCs, T cells and B cells is unclear. Furthermore, studies of Systemic Lupus Erythematosus (SLE) have provided evidence to suggest that self-antigens present in NETs will be retrieved and presented by plasmacytoid DCs, leading to the production of auto-antibodies (Lande *et al*, 2007). Similarly, Sangaletti *et al* (2012) have suggested that NETs can aid in the transfer of autoantigens to myeloid DCs, and Tillack *et al* (2012) provide evidence to suggest that NETs can increase T cell responsiveness and directly initiate T cell priming. Conversely a report from Barrientos *et al* (2014) suggests that NETs are instrumental in the downregulation of moDC LPS-induced activation. Further to

this, this study suggested that these moDCs had reduced capacity to stimulate T cell expansion and that the resulting T helper response was skewed towards a Th2 phenotype.

These studies provide evidence to suggest that NETs may have functions during *Leishmania* infection, beyond direct killing of promastigotes. With this in mind, it may be that during *L. mexicana* infection, NETs may aid antigen retrieval from parasites trapped in the filamentous extruded DNA, facilitating antigen uptake by macrophages or dendritic cells. In this capacity NETs could provide a bridge between innate immunity and the development of an adaptive immune response.

Evidently, the role of neutrophils during the initial stage of infection is complex. We have discussed the neutrophil as a potential primary host cell, how the cell may play a role in promoting further infection and how neutrophils contribute to extracellular killing. Further to this, neutrophils produce essential environmental cues to orchestrate immune cell recruitment to the site of infection. However, the extent to which cellular interactions between neutrophils and antigen presenting cells will affect the adaptive response during *L. mexicana* infection has yet to be defined. Determining whether neutrophils influence DC recruitment following *L. mexicana* infection (and their subsequent activation or migration) and how NETs might impact on resistance or susceptibility to disease will be crucial in defining the precise role of these cells *in vivo*.

1.5. The role of cytokines in *Leishmania* infection

Over the decades, *Leishmania* research has demonstrated the importance of various cytokines in determining the outcome of infection. Mossman *et al* (1986) originally

showed that cytokine expression impacts the type of T helper immunity that will develop during intracellular parasite infection. As we now know, Th1/Th2 responses directly correlate with healing and non-healing immunity, at least in *Leishmania major* infection (Heinzel *et al,* 1989). Significant evidence supports that production of the Th1 cytokines IFN-γ and IL-12 is essential for resistance to *L. major*, and implicates IL-4 as the inducer of Th2 non-healing immunity (Heinzel et al, 1989; Launois *et al,* 1995; Reiner *et al,* 1995). Additionally, Kostka *et al* (2009) demonstrated that IL-17/IL-23 and Th17 cells contribute to susceptibility in *L. major* infection and that production of these cytokines during infection will facilitate disease progression. Similarly, regulatory T cells (Tregs), that have been shown to accumulate in the dermis during lesion development, have been identified as an important source of IL-10 (Belkaid *et al,* 2001). As IL-10 has been implicated in aiding parasites survival, modulating antigen presentation and manipulating macrophage function this study provides evidence to suggest that Tregs are important for the parasite survival and progression of disease (Belkaid *et al,* 2001).

Whilst previous work has suggested that chronicity in *L. mexicana* infection is associated with the failure to mount an effective Th1-type response, evidence suggests that this attributable to a lack of IL-12 production during infection rather than a defective T cell response (Rodriguez-Sosa *et al*, 2001). However, a number of studies have demonstrated the importance of Th2 associated cytokines (like IL-4/IL-13) in directing lesion development during *L. mexicana.*

1.5.1. The role of IL-4/IL-13 in the development of infection

While there has been a great deal of research surrounding the role of Th2-associated cytokines in *L. mexicana* infection, the function(s) of IL-4/IL-13, and how these cytokines influence early lesion growth, has yet to be clearly defined. A number of workers have demonstrated that IL-4 is important in the development of disease following infection. Satoskar *et al* (1995) first demonstrated that following *L. mexicana* infection, C57BL/6 and 129 mice deficient in IL-4 production, do not develop progressive disease and the small lesions that do form contain fewer parasites. Following these findings, Alexander *et al* (2002) used BALB/c mice deficient in IL-4Rα (a component of the receptor for both IL-4 and IL-13) to investigate a putative role for IL-13 in disease promotion. Indeed, this group demonstrated that IL-4Rα$^{-/-}$ mice completely failed to develop lesions following infection. These findings suggested that IL-13 must have a contributory role in the development of chronic infection (Alexander *et al*, 2002).

1.5.2. Cell specific IL-4Rα-deficient mice aid in determining the function of IL-4/IL-13 during infection

The next step was to determine how IL-4/IL-13 can direct healing and non-healing immunity with relation to specific cell types. An early approach was to adoptively transfer IL-4$^{-/-}$ lymphocytes into SCID mice (which lack lymphocytes), which indicated that whilst a lack of lymphocyte-derived IL-4 was protective, a source of non-lymphocyte IL-4 plays a role in early development of the lesion (Satoskar et al, 1995).

More recently, a different approach has been utilised the Brombacher laboratory to create cell-specific IL-4Rα-deficient mice (using a cre/flox system) (reviewed by Hurdayal *et al,* 2014), allowing dissection of the role of IL-4/IL-13 in developing resistance and susceptibility to a range of pathogens (Holsher *et al,* 2006*;* Radawanska *et al*, 2007; Hurdayal *et al,* 2013). Using this approach, Bryson *et al* (2011) demonstrated that deletion of IL-4Rα specifically on macrophages and neutrophils ($LysM^{cre}IL\text{-}4R\alpha^{-/lox}$) does not affect the progression of disease following *L. mexicana* infection, whilst development of *L. major* in these mice is significantly impaired (Holsher *et al,* 2006). The same workers discovered that mice lacking IL-4Rα only on $CD4^+$ T cells ($Lck^{cre}IL\text{-}4R\alpha^{-/lox}$) demonstrated a sex-linked protective response, whereby female mice were fully resistant to *L. mexicana* infection and males developed small, but non-healing lesions. (Bryson *et al,* 2011). Interestingly, Radawanska *et al* (2007) found that both sexes of these $Lck^{cre}IL\text{-}4R\alpha^{-/lox}$ mice showed full resistance to *L. major* infection. Together, this work suggests important role for IL-4/IL-13 responsive T cells in control of progressive disease in both *L. major* and *L. mexicana* infection. However, importantly, in the context of *L. mexicana* infection, the initial development of the lesion does not appear to be influenced by these same T cells.

1.5.3. The importance of IL-4 for DC function

Paradoxically, whilst it is widely accepted that IL-4 is a stimulator of Th2 type differentiation during infection, early IL-4 has also been suggested to be crucial in priming for effective Th1 cell differentiation (Beidermann *et al.*, 2001; Ehrchen *et*

al., 2010). Early in *L. major* infection, during initial DC activation, IL-4 is required for optimal IL-12 production by DCs and helps to promote differentiation of IFN-γ-producing Th1 cells. Strikingly, administration of exogenous IL-4 can even reduce lesion growth, although only when given at the early stages of infection (8h after inoculation), suggesting that time-dependent production of IL-4 can determine disease outcome during *L. major* infection. In support of this work, Ehrchen *et al* (2010) suggest that keratinocyte-derived IL-4 is important in establishing a Th1 type response. Resistant mice induced higher levels of IL-4 during the first day of infection, blockade of which promotes development of a Th2-skewed response.

It should also be noted that IL-4 has been shown to have a role in the alternative activation of DCs (Cook *et al,* 2012). A body of evidence has demonstrated that IL-4 is required for the alternative activation of macrophages, stimulating the expression of RELM-α and Ym1 (archetypal markers of alternative activation) (reviewed by Gordon, 2003) and causes the suppression of T cell function, specifically during helminth infection (Pesce *et al*, 2009; Cook *et al,* 2011). Recent evidence suggests that similarly, IL-4 stimulates the expression of RELM-α in DCs, and thus has an influence on the DC's capacity to prime Th1/Th2 immune responses (Cook *et al,* 2012).

Interestingly, previous studies have described how vaccination with *L. major* loaded DCs in BALB/c mice can prevent the development of a lesion and subsequent tissue damage following *L. major* infection (Ramirez-Pineda *et al.,* 2004). Recently, it has been demonstrated that the success of this treatment this is dependent on IL-4 signaling. Indeed, in the absence of IL-4Rα, vaccinating with antigen-loaded DCs

did not prevent lesion formation. Therefore, this study identifies IL-4 signaling as an important pathway in preventing tissue damage in susceptible mice following *L. major* infection (Masic *et al,* 2012).

1.5.4. Importance of IL-4 in antibody production

IL-4 is a known modulator of B cell antibody production and various studies have shown that this cytokine can influence antibody class switching during infection, especially production of IgE and IgG1 (Finkelman *et al,* 1988). A body of work has established Th2 cells are the archetypal producers of IL-4, although Reinhart *et al* (2009) have demonstrated that follicular helper cells (Tfh) are the exclusive source of IL-4 in the LN. Therefore, it is important to consider that Tfh cells will be instrumental in the development of B cell follicles and germinal centres in the LN and will as such have great sway over B cell activation and subsequent antibody production.

Kima *et al* (2000) initially suggested that macrophage susceptibility to *L. mexicana* infection is dependent on antibody-meditated stimulation of FcR receptors and Colmenares *et al* (2002) demonstrated that without antibody there was reduced recruitment of macrophages and lymphocytes into the site of infection. These studies highlight a key role for antibody in the development of disease and a further role in regulating immune cell migration to the infection site. Further studies, largely carried out by the Buxbaum group, have suggested that FcγR is required for susceptibility, and that C57BL/6 mice without this shared Fc-receptor mount a protective response and resolve infections (Buxbaum and Scott, 2005). Kane and Mosser (2001) previously demonstrated that IgG1 can induce the production of IL-10 from

macrophages through the FcγRIII receptor and Chu *et al* (2010) determined that mice deficient in IgG1 were able to control disease more effectively than wild-type counterparts. Similarly, mice lacking FcγRIII (the Fc-receptor that binds IgG1) are resistant to *L. mexicana* infection, resolving infection and generating a stronger Th1 response (Thomas and Buxbaum, 2007). Together these studies suggest that IgG1 stimulation of FcγRIII is important for IL-10 production and susceptibility to disease following *L. mexicana* infection. Thus, it has been hypothesised that T cell-derived IL-4 production will drive parasite-specific IgG1 production by B cells, and this may be important in directing the outcome of infection through IgG1-mediated IL-10 production.

In support of this concept, recent studies of mice lacking IL-4Rα specifically on B cells (mb-1^{cre} x IL-4Rα$^{-/lox}$ mice) have shown that OVA immunisation results in altered antibody class switching by IL-4Rα-deficient B cells. These B cells failed to produce IgG1, and antibody production was skewed towards IgG2a and IgG2b isotypes (Hoving *et al*, 2012). Therefore, these animals provide an opportunity to definitively determine the role of IgG1 in the pathogenesis during *L. mexicana* infection.

1.6. Parasite virulence factors

So far, this Chapter has introduced how *Leishmania* spp. can manipulate and evade the host immune response. Importantly, a significant number of studies have identified some of the immunomodulatory molecules used by the *Leishmania* genus. Lipophosphogylcan (LPG), *Leishmania* associated LACK antigen, and the A2 gene cluster, are all important virulence factors that have been identified as contributing to

the evolution of this successful pathogen (Handman and Bullen, 2002; Gomez-Areazeas *et al*, 2011; Zhang and Matlashewski, 1997).

The expression and secretion of immunomodulatory compounds into the host environment is an important mechanism employed by the parasite to influence the host response. Indeed, *L. major, L. donovani,* and *L. aethiopica* promastigotes have all been shown to release LCF into their microenvironment, and evidence suggests that LCF stimulates the production of neutrophil-derived CXCL8 (van Zandbergen, 2002). In turn, this initiates a positive feedback loop that recruits more neutrophils and other leukocytes (expressing CXCL8 receptors – CXCR1 and CXCR2) to the site of infection, further contributing to inflammatory chemokine release.

Meanwhile, several studies have described how *Leishmania* can modulate host cell function from within the cell, whereby the parasite can influence key macrophage function by subverting phagosome-endosome fusion and antigen presentation (reviewed by Mosser and Miles, 2000). Parasite manipulation of parasitophorous vacuole maturation and biogenesis has been observed during *L. donovani* infection (Desjardin and Descoteaux, 1997) and recent work from Lippuner *et al* (2009) using GFP-tagged LPG has suggested that promastigotes can successfully use LPG to modulate phagosome maturation. By altering the course of phagosome-endosome fusion, the parasite has time to transform to the lysosome-resistant amastigote form, and thus resisting intracellular killing, but does this affect parasite antigen processing and presentation? To date, the antigen-processing pathway of *Leishmania* parasite has not been fully described. However, Kima *et al* (1996), have demonstrated that while promastigote antigens can be endogenously processed by the MHC Class II

pathway, amastigote antigen cannot and suggest that the change from promastigote to amastigote will provide a mechanism for evasion of MHC Class II presentation.

Certainly, there have been many studies investigating antigen presentation during *Leishmania* infection and the immunomodulatory role of parasite-derived molecules. Thus, it has long been established that surface presentation of MHC Class II can be suppressed by the parasite. In 1989, Reiner *et al* first demonstrated that *L. donovani* can suppress the surface expression of MHC Class II. Further to this, *L. amazonensis* amastigotes have been shown to be capable of directing the host macrophage to internalise and degrade MHC Class II molecules (de Souza Leao *et al,* 1995). However, in both these instances, it remained unclear as to how the parasite can have such an impact on host cell function.

Therefore, the characterisation of the cysteine proteases (CPs) of *L. mexicana* was a major step forward in understanding how this intracellular parasite might subvert the host cell. The CPs are parasite-derived proteases that are primarily expressed by the intracellular amastigote and have been shown to manipulate intracellular signalling pathways. Cameron *et al* (2004) have demonstrated that cytoplasmic NFκB and IκB are both degraded by cysteine protease B (CPB) in infected macrophages and that this modulation of intracellular signalling results in reduced IL-12 production. This work demonstrates that CPB can alter IL-12 production, essential for polarisation towards Th1 type immunity, and this suggests that cysteine proteases have the capacity to modulate the adaptive immune response. In support of this, further studies have demonstrated that *L. mexicana* derived CPs can influence the infectivity of metacyclic promastigotes and that this will limit disease. Parasites deficient in the CPB gene are not only less infective for BALB/c macrophages, but infection with

these parasites results in retarded lesion growth in BALB/c mice (Mottram *et al* 1996).

Whilst, it is clear that CPs have a profound influence on parasite function, how do these molecules influence adaptive immunity *in vivo*? Alexander *et al* (1998) demonstrated that during infection with CPB-deficient *L. mexicana,* production of Th2 cytokines was impaired with a concomitant increase in Th1 cytokines. Deletion of *cpa* or *cpb* in *L. mexicana* promastigotes can alter the development of protective immunity (Alexander *et al,* 1998). Strikingly, BALB/c mice infected with *Δcpa/cpb* parasites failed to form lesions at all. These results support that CPB-mediated disruption of NFκB signalling will result in impaired IFN-γ production following infection (Cameron *et al,* 2004). In addition, Pollock *et al* (2003) have suggested that CPB2.8, the most abundantly expressed form of CPB, can enhance IL-4 production during *L. mexicana* infection and injection with the purified protein during infection with *L. mexicana* will significantly exacerbate lesion growth (Pollock *et al,* 2003).

The cysteine proteases can also mediate the internalisation and degradation of MHC Class II molecules during trafficking to the macrophage cell surface (de Souza Leao *et al*, 1995), but the effects of CPB2.8 may not just be limited to intracellular activities. In fact, Pollock *et al* (2003) and others (Ilg *et al,* 1994) have demonstrated that CPB can be detected in the extracellular milieu. The mechanism by which CPB is secreted from the parasitophorous vacuole has not yet been fully defined. CPB is found at high concentrations in the extracellular milieu in established lesions (Ilg *et al*, 1994), a process that could come about via the lysis of infected host cells (Mottram et al 2004) possibly by physical disruption of the cell following amastigote replication, or perhaps facilitated by the activity of an identified

cytolysin, Leishporin (Noronha *et al,* 2000; Almeida-Campos and Horta, 2000; Almeida-Campos 2013).

Taken together these studies demonstrate that parasite derived CPs can have a range of activities that may influence host immunity, including modulating trafficking of MHC Class II molecules, suppressing intracellular NFκB signalling, impairing the development of Th1 type immunity and/or enhancing Th2 associated cytokine production. Therefore, it is clear that CPs, especially CPB can very successfully sustain *Leishmania* infection, and promote chronic infection. However, the mechanism(s) that CPB employs to manipulate the host cell is not fully defined. As discussed the protease can not only influence signalling and trafficking within the infected cell, but may also have an unknown extracellular function and it is unknown whether the protease can influence the recruitment and migration of immune cells during *L. mexicana* infection. As previous reports have identified dysregulation of DC migration during *Leishmania* infection, perhaps CPB could have a role in the modulation of DCs chemotaxis following *L. mexicana* infection?

1.7. Significance of understanding the host immune response to *Leishmania*

Understanding the fundamental differences in the host immune response that leads to either protection or susceptibility to different *Leishmania* species and strains is essential for the development of disease prevention methods. With parasite resistance to miltesophine and other therapeutic treatments on the increase (Croft *et al*, 2006), unravelling the complexities of the host-parasite interaction may provide the basis

for development of more efficacious drugs or provide novel vaccines or targets for immunotherapy in the future (Nagill and Kaur, 2011). The recent success using TLR7/8 agonist drugs as adjuvants for *Leishmania* vaccines, suggest that protective immunity is achievable if the immune response is adequately and appropriately stimulated (Zhang and Matlashweski, 2008). Conversely, by studying the immune response, and its failures during *Leishmania spp.* infection, we can hope to answer more fundamental questions about the immune response during infectious and non-infectious chronic disease.

1.8. Aims and objectives

The complex interactions between immune cells, their secreted factors and parasite-derived products will all contribute to the development of the host immune response and determine whether protection or susceptibility prevails. *Leishmania mexicana* is able to modulate a number of key factors directing the immune response accordingly and, unlike *L. major*, can establish chronicity across a range of mouse genetic backgrounds. The development of chronic infection may be associated with the ability to evade effective capture, processing and presentation of parasite antigen and the subsequent activation of naïve T cells and B cells. However, exactly how *L. mexicana* can manipulate these early stages of the host response that are so important in influencing immunity, is not fully understood. As one of the essential steps in the induction of the adaptive immune response is the recruitment and migration of DCs, **the overall aim of this thesis was to establish whether *Leishmania mexicana* can influence cellular migration and investigate the parasite- and host-derived factors that influence DC function.** Specifically, I sought to apply a range of *in vitro* and *in vivo* approaches to address the following key objectives:

- To establish whether *L. mexicana* modulates DC migration *in vitro* and *in vivo* and explore the implications for induction of adaptive immunity?
- To explore whether stimulation of DC migration during initial *L. mexicana* infection could alter the outcome of disease.
- To determine the role of the virulence factor cysteine protease B, if any, in influencing DC migration during *L. mexicana* infection?
- To investigate the role of neutrophils and NETs in the development of chronic infections with *L. mexicana*.
- To investigate the importance of IL-4Rα signaling in B cells and DCs following *L. mexicana* infection.

The key finding of this thesis is that *L. mexicana* is able to abrogate DC migration in early infection, in part through CPB-mediated degradation of CCL19. Additionally, neutrophils and their NETs are also shown to be important for effective DC function, as may the actions of IL-4. Overall this study has important implications for understanding the early immune response to infection, how this defines the development of chronic disease and how we may develop new therapies for intracellular parasite infections.

Chapter 2. Materials and methods

2.1. Animals

BALB/c, C57BL/6 and Ly5.1 congenic mice were all bred and maintained in the Biological Procedures Unit at the University of Strathclyde, Glasgow. OT-II mice, the $CD4^+$ T cells of which express a transgenic T cell receptor specific for ovalbumin (Barnden *et al,* 1998), were originally purchased from Charles River Labs and maintained as an in-bred colony in the BPU. Peptidylarginine deiminase 4-deficient ($PAD4^{-/-}$) mice, which lack the ability to produce NETs (Hemmers *et al,* 2011) were a generous gift from Dr Kerri Mowen, Scripps Institute, California, USA and were maintained with their wild-type counter-parts in an air-filtered isolator system. The IL-$4Ra^{-/-}$, $mb1^{cre}$ mice with IL-$4R\alpha^{-/-}$ (Hoving *et al,* 2012), and $CD11c^{cre}$IL-$4Ra^{-/-}$ (Hurdayal *et al,* 2013) were obtained from Prof Frank Brombacher at the University of Cape Town and were maintained in isolation, as above. All animal maintenance and procedures were carried out in accordance with Home Office and University of Strathclyde Animal Welfare and Ethical Review Board regulations and experimental design and reporting adhere to the ARRIVE guidelines.

2.2. Mouse treatments

Mouse TLR-7 agonist, 4-Amino-2-(ethoxymethyl)-a,a-dimethyl-1*H*-imidazo[4,5-c]quinoline-1-ethanol (S 28463) also known as R-848 (Enzo Life sciences) was used to stimulate DC activation and migration. For therapeutic administration mice were subcutaneously injected with 25 ng of R-848 (50 µl injection volume) in the footpad, this dosage was chosen in line with the results described in Figure 4.2. R-848 was either injected once, 18 h post *L. mexicana* infection to correspond with the

height of DC migration, described by Tal *et al* (2011) or at 0 h, 1 week and 2 weeks post infection.

Anti-neutrophil (NIMP-R14) antibody, a generous gift from Prof. Fabienne Tacchini- Cottier, UNIL, Switzerland was used to mediate depletion of Ly6-C^+ and Ly6-G^+ cells. This antibody effectively mediates depletion of neutrophils from 6 hours post-administration. Mice were injected i.p. with 250 μg (200 μl injection volume) of the antibody (in line with previous work carried out by Tacchini-Cottier *et al,* 2000).

2.3. MPO bioluminescence imaging

Luminol (200 mg/kg) was injected i.p. to detect MPO activity, an indirect measure of neutrophil infiltration, as described by Gross *et al* (2009). Optimal exposure time was determined by time course experiment. 5 minutes post-luminol injection the animals were imaged using a medium binning setting and 5 minute exposure time using the IVIS system. The total flux (photons/s) was used to quantify MPO activity in specified regions of interest (ROI) using a Xenogen intravital imaging system (IVIS).

2.4. *Leishmania* parasite culture and soluble Leishmania antigen (SLA) preparation.

Wild-type *Leishmania mexicana* (MYNC/BZ/62/M37) promastigotes were maintained *in vitro* at 27°C in HomeM medium supplemented with 10% foetal calf serum (both PAA Laboratories UK, Ltd), 100 μg/ml streptomycin, 100 U/penicillin (both Cambrex, UK), and 2 mM L-glutamine (Sigma, UK) as described previously by Mottram *et al* (1999). The parasites were passaged on day 7 of culture. *Cpb-*

deficient promastigotes were maintained in the same manner with the addition of Hygromycin B (Sigma, UK), final concentration of 1 μg/ml, in culture medium (Cameron *et al,* 2004).

SLA was prepared by as described previously Scott *et al,* (1987) by resuspending a confluent flask of parasites in 1 ml of PBS and flash-freezing the suspension in liquid nitrogen. The suspension was thawed at 60°C, aspirated 5 times with a 22 g needle, re-frozen in liquid nitrogen and freeze-thawed a further 4 times before filtering through a 0.22 μm filter. SLA concentration was determined using a Bradfords' protein assay (BioRad) as per manufacturer's guidelines. Briefly, 200 μl of BioRad (BioRad, UK) (1:5 dilution in ddH_20) was added to a 10 μl sample of SLA or 10μl of bovine serum albumin (BSA) (Sigma, UK) standards. The reaction was incubated at room temperature for 10 minutes and a spectrophotometer was used to measure absorbance at 570nm wavelength. The concentration of the sample was calculated from the standard curve values.

2.5. Leishmania infections

To initiate infections, promastigotes were harvested from culture flasks, washed in fresh HomeM medium and resuspended in PBS at the desired concentration. Amastigotes were obtained from the lesions of infected animals, and were washed, counted, resuspended to the desired concentration in PBS and used immediately.

To initiate short-term infections for imaging or flow cytometry experiments, parasites were diluted to either $5x10^7$ metacyclic promastigotes/ml (unless otherwise stated) for intradermal injection into the ear pinna (10 μl injection volume), or to

$1x10^7$ parasites/ml for subcutaneous inoculation into the footpad (50 µl injection volume) as described in Charmoy *et al* (2010). For long-term monitoring of *Leishmania* lesion development, infection was initiated by subcutaneous injection of either $5x10^7$ promastigotes/ml (for footpad infections) or $2.5x10^7$ amastigotes/ml (for rump infections at the base of the tail) as described by Alexander *et al* (1998). Lesion development was monitored by weekly measurement of footpad thickness using a spring-loaded Mitutoyo thickness gauge calliper. Lesion size was calculated by subtracting the thickness of the contralateral footpad from the thickness of the infected footpad. Rump lesion size was taken to be the diameter of the lesion measured by sliding calliper.

In some of these experiments, parasites were fluorescently labeled using 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies, UK), as per manufacturer's guidelines. CFSE was added to parasites (at $1x10^7$ parasites / ml) to a final concentration of 1 µM in PBS. The parasites were incubated for 10 minutes at 37°C, washed in twice in cRPMI twice and used immediately.

2.6. Murine bone-marrow derived dendritic cell (BmDC) generation and culture.

BmDCs were generated from bone marrow of 8-10 week old BALB/c, C57BL/6, Ly5.1 or IL-4R$\alpha^{-/-}$ mice, as previously described by Lutz *et al* (1999). The tibia and femurs were collected and the epiphyses were removed from the bones. Using a 25 g needle, bone marrow was flushed out and disaggregated to form a single cell suspension. The cells were suspended in 'complete RPMI' (cRPMI), containing Roswell Park Memorial Institute (RPMI) 1640 medium (PAA Laboratories Ltd,

UK), 10% Heat-inactivated foetal calf serum (Biosera), 100 μg/ml streptomycin, 100 U/ml penicillin (both Cambrex, UK), and 2 mM L-glutamine (Sigma Aldrich, UK). For generation of DCs, cRPMI was supplemented with 10% medium derived from x63 cells expressing GM-CSF (a generous gift from Prof B Stockinger, MRC labs). Cells were seeded into bacteriological petri dishes and incubated for 7 days at 37 °C, with 5 % CO_2. On day 3 of culture, an additional 10 ml of fresh cRPMI supplemented with 10% GMCSF was added to each dish and on day 5 of culture half of the volume was replaced with fresh media. The differentiated bmDCs were harvested on day 7 of culture and replated into GM-CSF-free media for further use. Cells were routinely 70-90 % $CD11c^+$ by flow cytometry.

After generation of bmDCs, medium was removed and the culture plate rinsed with cold sterile HBSS (Life Technologies, UK). Adherent cells were gently agitated from the petri dish using plate scraper. The cells were then washed, counted and resuspended in cRPMI to the appropriate concentration, and plated at a concentration of 5-10x10^5 cells/ml into 6 well or 12 well plates and allowed to settle overnight before use.

2.7. Stimulation of bmDCs

In some experiments, bmDCs were exposed to *L. mexicana* metacyclic promastigotes. In such experiments, parasites were prepared as described above and added to DCs, usually at a 5:1 ratio (ie. 5 parasites per DC; unless otherwise indicated) 24 h prior to cell harvest. For most experiments, stimulation with either lipopolysaccharide (LPS (*E. coli* 0127 (Sigma, UK); 1 μg/ml final concentration) or resiquimod (R-848; 100 ng/ml final concentration, as described by Burns *et al*

(2000)) was carried out 18 h prior to cell harvest. In addition, recombinant CPB2.8, a generous gift from Gareth Westrop, University Strathclyde, UK, was used treat DCs. DCs were first LPS stimulated then treated with 20 μg/ml. In all experiments, bmDCs were washed at least twice in cRPMI before use in subsequent *in vitro* or *in vivo* assays.

For tracking bmDCs *in vivo*, cells were labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Life Technologies, UK). CFSE was added to cells (at $1x10^7$ cells/ml) to a final concentration of 1 μM in PBS as per manufacturer's guidelines. The cells were incubated for 10 minutes at 37°C, washed in twice in cRPMI twice and resuspended to transfer $1x10^5$ cells in 10 μl of injected volume (for intradermal ear injections) or $1x10^6$ cells in 50 μl for subcutaneous footpad injections. In some experiments, DCs were pulsed with 1 mg/ml $OVA_{323-339}$ peptide (ProImmune) for 4 h prior to transfer, as described in Pape *et al* (1997).

2.8. 3D chemotaxis migration assay

All reagents were gas and temperature equilibrated to 37°C and 5% CO_2 by placing in the incubator overnight. Parasite-exposed, or control bmDCs were washed, resuspended to a final concentration of $3x10^6$ bmDCs/ml in a mixture of 0.25% $NaHCO_3$, 1x MEM and 1.5 mg/ml final concentration of bovine collagen I (all Sigma-Aldrich) and mixed gently avoiding the formation of air bubbles. The gel was loaded into a μ-Slide Chemotaxis 3D chamber (Ibidi), allowed to polymerise and then a soluble chemokine gradient was established across the gel by loading 1.25 μg/ml CCL19 (R&D Systems) diluted in cRPMI to the left chamber and cRPMI in the right, in accordance with manufacturers instructions.

Time-lapse imaging was performed in a heated environmental chamber built around an inverted Axioplan epifluorescent microscope, acquiring brightfield and/or fluorescence images using a x 4 objective lens (Pixel size: 3.14 pixels/μm) at 2 min intervals for 4 hours.

In some experiments CPB2.8 was used to pre-treat. CCL19 (0.1 μg) was mixed with CPB2.8 (1.936μg) and incubated at 37°C for 15 minutes. The pre-treated CCL19 was then used in the 3D chemotaxis assay.

2.9. Flow cytometry

Aliquots of 1–10 x10^5 cells in 12 x 75mm polystyrene tubes (BD Bioscience, UK) were washed twice and resuspended in 100 μl FACS Flow along with the appropriate fluorochrome-conjugated antibodies (Table 1). Cells were incubated at 4°C for 30 minutes, washed and resuspended in 300 μl for acquisition (cells were incubated for a further 30 minutes with streptavidin conjugated antibody if required). Data was acquired using a BD FACSCanto flow cytometer running FACSDiva software (BD Bioscience) and analysed using FlowJo software (FlowJo LLC). Prior to running all samples, voltages for the photomultiplier tubes were adjusted such that cells could be gated on the basis of their FSC (forward scatter) and SSC (side scatter) profiles and unstained and single-stained cells or CompBeads (BD Bioscience) used to set compensation values.

Cell Marker	Primary antibody	Clone	Isotype Control
Fc Block	Anti-mouse CD16/32 FcBlock	93	-
CD11c	Anti-mouse CD11c PE Anti-mouse CD11c APC Anti-mouse CD11c biotin	N418 HL3	Armenian Hamster IgG cocktail-PE Armenian Hamster IgG cocktail-APC
IA-IE	Anti-mouse MHC Class II FITC	M5/111 4.15.12	Rat IgG2a- FITC
F4/80	Anti-mouse F4/80 APC	BM8	Rat IgG2a- APC
Ly6G	Rat anti-mouse Ly6G PE	1A8	Armenian Hamster IgG cocktail-PE
CD40	Anti-mouse CD40 APC	IC10	Armenian Hamster IgG cocktail-APC
CD45.2	Anti-mouse CD45.2 APC	104	Armenian Hamster IgG cocktail-APC
CCR7	Anti-mouse CCR7 biotin	-	-
Vα2	Anti-mouse Vα2 TCR-allocyanin	-	Armenian Hamster IgG cocktail-APC
Vβ5	Anti-mouse Vβ5 TCR	-	-
CD4	Rat anti-mouse CD4 PerCP	-	Conjugated beads
Ly5.1	Anti-mouse CD45.1 PE Anti-mouse CD45.2 APC	A20 104	Armenian Hamster IgG cocktail-PE Armenian Hamster IgG cocktail-APC
B220	Anti-mouse B220 450	RA3-6B2	Armenian Hamster IgG cocktail-APC
LYVE-1	Anti-mouse Lyve-1 eFluor 660	A2Y7	Armenian Hamster IgG cocktail-PE

Table 2. Flow cytometry antibodies used for cell marker identification. All antibodies were supplied by eBiosciences.

2.10. RNA extractions & Quantitiative PCR

BmDCs were cultured *in vitro* as detailed previously (Section 2.6). Cells were harvested, mRNA was extracted using a RNeasy mini kit (Quaigen). The mRNA was then reverse transcribed to synthesise cDNA, using a high capacity RT-Kit (Life Technologies) and quantitative RT-PCR was performed using a StepOne Plus Real time PCR system (Life Technologies). Taqman Gene expression assay for both 18S (Hs99999901_s1) and CCR7 (Mm0131785_m1). The results were calculated using the comparative threshold (CT). Results were normalised to 18S housekeeping gene endogenous control and results were presented as fold increase relative to unstimulated DCs (value of 1).

2.11. Cell isolation

To isolate cells from the dermal infection site, ears were removed 2 h or 24 h post-intradermal injection of *L. mexicana* promastigotes and placed into cRPMI. Naïve tissue was used a negative control sample, whilst mice were challenged with 60 μg of carrageenan (Sigma-Aldrich; dissolved in PBS) as a positive control.The ear dermis was split into dorsal and ventral sheets (as described by Belkaid *et al*, 1996), and the 37°CFor lymph node cell isolation, LN were collected into a small amount of cRPMI and disrupted using the plunger of a 1 ml syringe and Nitex mesh (Cadish & Sons). The remaining tissue was treated with collagenase IV (0.5 mg/ml; Sigma) in PBS at 37°C for 25 minutes to dissociate tissue and liberate adherent cells. The collected cell suspension was then washed and prepared for flow cytometry.

2.12. Adoptive transfer procedure

The lymph nodes and spleen were recovered from donor OT-II mice (containing OVA-specific $CD4^+$ T cells), homogenised to make a single cell suspension (as described above), counted and a sample analysed by flow cytometry to determine the proportion of OVA-specific T cells (identified by Vα2 and Vβ5 TCR expression) as described in Pape *et al* (1997). Cells were CFSE-labelled and $3x10^6$ OVA-specific T cells injected intravenously into C57BL/6 recipient mice. $1x10^6$ antigen-pulsed bmDCs were injected into the footpad of recipients, as described above. After 72 hours, the draining popliteal lymph node was removed, homogenised and prepared for flow cytometry to quantify the proportion of OVA-specific T cells as well as analysis of cell division based on CFSE intensity.

2.13. Whole-mount fluorescent staining of ear tissue.

Tissues were first fixed by immersing in 4% paraformaldehyde at 4°C for 24 hours. Lymph nodes undergoing fixation remained whole, whilst the ear dermis was split into dorsal and ventral sheets. Tissue was transferred to permeabilisation buffer (1% BSA; 0.3% TritonX-100; 0.2% sodium azide (all Sigma-Aldrich) in PBS) and incubated at 4°C for 1 hour. This step was repeated twice before the tissues were immersed in permeabilisation solution with the appropriate antibodies (anti-LYVE-1 eF660/anti-IA-IE FITC) at a concentration of 2 μg/ml and incubated at 4°C for 24 hours. After staining, the samples were rinsed in PBS, transferred to fresh permeabilisation buffer and incubated for 4°C for 20 minutes. This step was repeated twice more and the sample was rinsed in PBS before mounting on a glass slide with Vectashield mounting medium (Vector labs) before imaging with confocal microscopy as described in Cummings *et al* (2009). Images were acquired

using a Leica SP5 laser-scanning confocal microscope. Three fields of view/sample was routinely acquired. Each z-stack comprised of 1.98 μm z-steps, over a total depth of 20-25 μm. (Pixel size: 0.758 μm). All images are representative of 3 or more replicates and are shown as z-compressed images.

2.14. Cryosectioning of tissue samples and IHC staining

Tissue was immersed in OCT freezing medium (VWR chemicals), frozen and stored at -80°C. The sample was then cut into 8 μm thin slices using a Shandon cryostat and mounted onto Superfrost thermoglass slides. Care was taken to avoid freeze-thaw of the tissue during processing. Slides were stored at -20°C before use.

For histological staining, slides were brought to room temperature and an optimised haemotoxylin and eosin staining procedure was followed. Briefly, slides were fixed using acetone/ethanol mix (75%/25%) for 10 minutes and allowed to dry. The slides were rehydrated for 10 minutes in 0.05% Tween (Sigma, UK) solution (in PBS) on a shaker. Slides were immersed in Haematoxylin stain (Shandon) for 5 minutes, then washed in running water for 5 minutes. Slides were then immersed in Scots Tap Water Solution (STWS) for 3 minutes and washed in running water for 5 minutes. The slides were transferred to Eosin solution (Shandon) for 3 minutes and washed in running water for 2 minutes. Slides were then briefly immersed in 70%, and then 95% ethanol. Slides were then immersed in 100% ethanol for 5 minutes to ensure full dehydration. Slides were cleared in Histoclear for 5 minutes and were imaged using and upright Nikon Eclipse 50*i* microscope.

For immunofluorescent staining, slides were defrosted and transferred to a humidified chamber. The samples were fixed in 4% paraformaldehyde (Sigma, UK)

for 30 minutes at room temperature and blocked with 1% BSA (in PBS) for three 5 minute incubations. The sample was then incubated for 20 minutes with FcBlock (1:400), rinsed and stained using anti-B220-450 and anti-LYVE-1-eF660 (final concentration of 2 μg/ml). Slides were washed a further three times, mounted in Vectashield and imaged by confocal microscope.

2.15. Gel analysis

CCL19 (121 ng), CPB2.8 (2.34 μg) or a combination of both (121ng CCL19 + 2.34μg CPB2.8) and were mixed with 3.25 μl loading buffer (Amresco), denatured and loaded into separate wells of 15% Glycine NEXTgel (Amresco). Amersham Low rainbow molecular marker (GE) was chosen as an appropriate marker for small proteins/fragments. The gel was run in a Biorad Mini protein III tank, in Next Gel running buffer at 150 Volts for 90 minutes, in accordance with manufacturers guidelines. The gel was then stained using Silver Stain Kit (Pierce; Thermo Scientific) and was developed for a maximum of 30 seconds.

2.16. Enumeration of lesion parasites

Lesions were aseptically excised, and homogenised through Nitex membrane to create a single cell suspension in HomeM medium. Cell suspensions from the lesions were prepared in the same volume of medium (6.4 ml), and 100 μl of that suspension was used to perform 2-fold dilution, it was possible to calculate the number of parasites (log_2) in the lesion cell suspension. The parasites were plated by serial 2-fold dilution in HomeM medium across 96-well flat-bottomed plates. The parasite dilutions were incubated for 7-10 days at 27°C to allow sufficient time for parasite replication. Through microscopic examination of samples it was

possible to determine that the lowest dilution at which no parasites had grown, i.e. the limiting dilution.

2.17. Splenocyte stimulation for quantification of cytokine production

Spleens were removed from infected mice, homogenised to form a single cell suspension and stimulated with SLA (as described by Satoskar *et al,* 1995). Flat-bottomed 96-well plates were filled with 100 µl SLA at a concentration of 50 µg/ml, 20 µg/ml or 10 µg/ml (unless stated otherwise), ConA (5 µg/ml) or complete RPMI. 100 µl of each splenocyte suspension was added to appropriate wells and incubated at 37°C for 72 h, after which, the supernatants were harvested and stored at -20°C until quantification of cytokine concentration by capture ELISA.

Details of the antibodies, recombinant standards and dilutions used in ELISAs are listed in Table 2.2. To quantify cytokine production, 96-well ELISA plates were coated with 50µl capture antibody (2 µg/ml) and incubated overnight at 4°C. The plates were washed three times in wash buffer (0.05% Tween 20 in pH 7.4 PBS). 30 µl of the supernatants or recombinant standards were added to the appropriate wells and incubated a 37°C for 2 h. Plates were washed as described above. 50µl of detection biotinylated antibody was then added for 1 h. Plates were washed as described above. 50 µl Streptavidin-AKP was added for a 45 minute incubation period at 37°C and after thoroughly washing the plates, 50 µl pNPP substrate was added. The absorbance was read at 405nm using a SpectraMax spectrophotometer. Concentration of cytokine was determined by comparison with the standard curve.

2.18. Quantification of IgG1 and IgG2a antibody titre

Blood was collected via cardiac puncture and serum was obtained after microcentrifugation of the clotted blood at 13,000 rpm, as described by Satoskar *et al,* 1995. The serum was then tested for *L. mexicana* specific-IgG1 and IgG2a. Flat-bottomed 96-well plates were coated with 100 µl of SLA for 24h at 4°C. The plates were washed three times in wash buffer (0.05% Tween 20 in pH 7.4 PBS) and were blocked using 4% milk powder incubated for 1 h at 37°C Serial dilutions of serum were added to appropriate wells and incubated for 1 h at 37°C. Plates were again washed and the bound *L. mexicana*-specific antibodies were detected using a HRP-conjugated goat anti-mouse IgG1 or IgG2a and plates developed with TMB substrate (KPL). The cataylsis of HRP was stopped when the colour change was sufficient for detection, by adding 10% H_2SO_4. The absorbance was measured at 450 nm using a spectrophotometer. The dilution at which there was a distinct reduction in optical density (to background level) was identified as the end-point. Results are presented as reciprocal end-point dilution.

Cytokine	Recombinant protein standard (Starting concentration)	Capture antibody (Clone)	Primary antibody	Biotinylated antibody (Clone)	Streptavidin conjugate (Clone)	Substrate
IFN-γ	rIFN-γ (2 ng /ml)	Rat anti-mouse IFN-γ (R4-6A2)	-	Biotin Rat anti-mouse IFN-γ (XMG1.2)	AKP	pNPP (Sigma, UK)
IL-5	rIL-5 (5 ng /ml)	Rat anti-mouse IL-5 (TRFK5)	-	Biotin Rat anti-mouse IL-5 (TRFK5)	AKP	pNPP
IL-10	rIL-10 (10 ng /ml)	Rat anti-mouse IL-10 (JES5-2A5)	-	Biotin Rat anti-mouse IL-10 (SXC.1)	AKP	pNPP
IgG1	N/A	SLA	Goat anti-mouse IgG1 HRP (Southern Biotech)	-	-	TMB (KPL)
IgG2a	N/A	SLA	Goat anti-mouse IgG2a HRP (Southern Biotech)	-	-	TMB

Table 3. Cytokine and antibody ELISA antibodies used throughout. Antibodies supplied by BDPharmingen, unless otherwise stated.

2.19. Imaging and analysis

Images were analysed using Volocity Software. Initially (if required), a region of interest (ROI) was created and this area was then further analysed. All confocal images were contrast enhanced when necessary, and are presented as z-compressed images throughout where appropriate.

For cell tracking of bmDCs in the 3D chemotaxis assay an optimised protocol was developed where individual cells were identified using the "Find objects" function using percentage intensity. Objects smaller than 5μm^2 were excluded. Objects were then tracked over at least 121 time-points using the object tracking function. The automated object tracking function was set to join broken tracks, with a maximum distance between objects of 3-5 μm to ensure accurate tracking of cells.

For colocalisation of CFSE cells with LYVE-1 eF660 labelled lymphatic vessels (LV). Images were analysed in 3D opacity mode. The degree of colocalisation between green DCs and red LVs in acquired images was quantified by calculating the proportion of green voxels (three-dimensional pixels) which contain above-threshold intensity of red voxels. This is known as the co-localisation co-efficient or overlap co-efficient.

For LN reconstruction, the entire area of 8-10 μm thick cyrosections of LN was imaged sequentially using the stitch and tile function of Leica SP5 software. (Pixel size: 1.25 μm/pixel). Stitched images of each slice were imported and the Z-step size was set a 10 μm for reconstruction in Volocity software and images of each LN section were used to form a reconstruction of the entire LN. CFSE labelled DCs were identified using percentage intensity (green channel).

2.20. Statistical Analysis

Data was analysed using GraphPad Prism software. Unpaired T-tests, Mann-Whitney, 1-way and 2-way ANOVA with Bonferroni post-tests were used to determine the significance of the generated data. Throughout * = $p<0.05$, **=$p<0.01$, ***=$p<0.001$.

Chapter 3. Dendritic cells fail to migrate into the lymphatic vessels and to the lymph node during *Leishmania mexicana* infection

3.1. Introduction

Infection with *L. mexicana* is characterised by the formation of chronic cutaneous lesions in most mouse strains (Rosas *et al*, 2005). This differs from *L. major* infections where the majority of mouse strains are resistant (Alexander and Kaye, 1985). It is hypothesised that the *L. mexicana* non-healing phenotype can be attributed to the absence of a strong Th1 type immune response (Rodriguez-Sosa *et al,* 2001). However, it remains unclear as to how the parasite can modulate the host response in this fashion.

Chemokines and cytokines have been shown to orchestrate the recruitment and migration of immune cells during both steady and infection states (reviewed by Teixiera *et al,* 2008; Esche *et al,* 2005). A balance of chemokine and chemokine receptor expression can ensure the orchestrated migration of innate immune cells, phagocytes and T cells, to provide continual surveillance for potentially harmful pathogens (Moseman and Williman, 2004). The kinetics of immune cell migration during infection has been shown to directly influence antigen presentation and generation of an effective memory T cell response (Muller *et al*, 2001). Therefore, the cellular dynamics during the initial infection stage are instrumental in determining disease outcome during *L. mexicana* infection. Characteristically, as has been demonstrated in a number of studies particularly using *L. major,* neutrophils are the first cells to reach the infection site, with monocytes and macrophages following closely thereafter (reviewed von Stebut, 2007). A similar sequence of events has recently been shown to take place following infection with *L. mexicana* (Hurrell *et al*, 2015). Following the initial cell mediated response, dendritic cells (DCs) infiltrate the site of *L. major* infection (Ng *et al,* 2008) although this process appears by

comparison to be significantly inhibited following infection with *L. mexicana* (Petritus *et al*, 2012; Hurrell *et al*, 2015).

Recent evidence suggests that functional DC chemotaxis is essential for parasite antigen presentation and the subsequent priming of a T cell response during *Leishmania* infection (Ato *et al*, 2006). This process is largely controlled by CCR7 expression on the surface of DCs and expression of CCL19/CCL21 by the lymphatic vessel endothelium (Comerford *et al,* 2013). To this end, Ato *et al* (2002) present convincing evidence to suggest that the chronicity of *L. donovani* infection is related to reduced interactions between *L. donovani* antigen presenting DCs and naïve T-cells in the spleen. The authors suggest that this is due to downregulation of CCR7 on the surface of DCs (Ato et al, 2002). In addition, it has recently been demonstrated that *L. mexicana* modulates moDC migration during infection (Petritus *et al* 2012; Hurrell *et al* 2015). Petritus *et al* (2012) show that fewer moDCs were present in the draining LN after infection compared with *L. major* infection. It can be hypothesised that the failure of migration of moDCs from the site of infection and into the draining LN could be a result of the regulation of chemokines or their receptors, including CCR7. Thus, modulation of this receptor during *L. mexicana* infection could be a major contributing factor to the formation of chronic infection.

Further to this, the progression of leishmaniasis is dependent upon the interactions between antigen presenting cells and naïve T cells in the lymph nodes and spleen (Ato et al, 2002: Ato *et al,* 2006). The diminished dendritic cell migration and altered chemokine profile described by Ato *et al* (2002) and Petritus *et al* (2012) suggest that a dysregulation of this process will contribute to the chronicity of cutaneous as well as visceral leishmaniasis.

In this chapter it was investigated whether *L. mexicana* modulates DC migration *in vitro* and *in vivo* and the implication of this for the induction of adaptive immunity was explored.

3.2. Results

3.2.1. *Leishmania mexicana* fails to activate DCs and modulates CCR7 expression.

The expression of CCR7 on the surface of dendritic cells (DCs) is important for the migration of these cells towards lymphatic vessels (Dieu *et al,* 1998; Jang *et al,* 2006) and in mice lacking CCR7, DCs fail to accumulate in the draining lymph node (Forster *et al,* 1999; Ohl *et al,* 2004). As has been previously discussed, disruption of CCR7 during *L. donovani* infection can abrogate DC migration and reduce DC-T cell interactions (Ato *et al*, 2006). Thus, it is important to determine if modulation of this receptor occurs during *L. mexicana* infection and if so, whether this is important in the development of chronic infection. Therefore, the expression of this receptor on the surface of bone marrow-derived DCs (bmDCs) exposed to WT *L. mexicana* metacyclic promastigotes was investigated.

Using flow cytometry, $CD11c^+$ bmDCs were identified and the proportion of CCR7-expressing cells quantified by determining the proportion of cells showing staining above that of isotype controls (Figure 3.1A). Whilst a small percentage of unstimulated bmDCs expressed CCR7, stimulation of cells with the TLR ligand, LPS, resulted in a significant increase in the proportion of CCR7-expressing cells. Strikingly, exposure to *L. mexicana* (at a 5 to 1 ratio of parasites to DCs) failed to induce upregulation of CCR7 expression by bmDCs, resulting in a small $CCR7^+$

population similar to that displayed by the unstimulated DCs. Further to this, subsequent LPS-stimulation of *L. mexicana* exposed bmDCs failed to boost CCR7 expression, suggesting that *L. mexicana* abrogates DC activation and/or suppresses CCR7 surface expression by bmDCs.

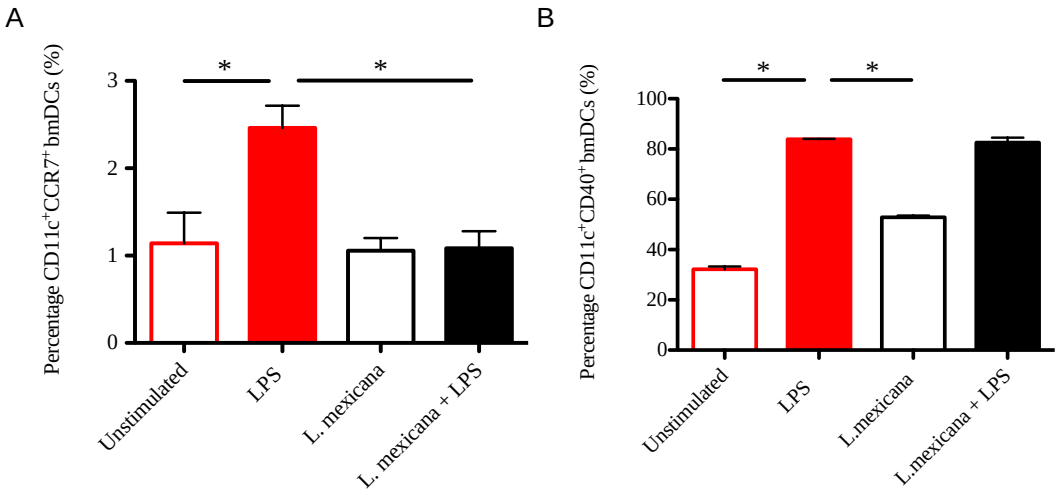


Figure 3.1. Protein expression of CCR7 and CD40 by bmDCs is regulated by *in vitro* exposure to *Leishmania mexicana*. Bone marrow-derived DCs were exposed to stationary stage metacyclic *L. mexicana* promastigotes at a 5:1 ratio of parasites to DCs and/or stimulation with LPS (1 µg/ml). After 18 hours of co-culture, cells were analysed by flow cytometry and the proportion of $CD11c^+$-gated cells expressing A) CCR7 or B) CD40 determined. Results show the mean ± SEM of 3 experiments each comprising 3 replicate samples. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. *=$p<0.05$.

To investigate this further, the effect of *L. mexicana* on the expression of the co-stimulatory molecule CD40 was assessed using flow cytometry. CD40 is a known

DC activation marker induced by TLR activation (Ma *et al,* 2009), and is a surface receptor essential for the activation of T cells. As expected LPS activation significantly increased the frequency of CD40-expressing bmDCs relative to resting cells (Figure 3.1B). DCs exposed to *L. mexicana* alone showed similar expression levels to unstimulated DCs. However, CD40 expression was effectively upregulated by parasite-containing bmDCs upon additional LPS activation. This would suggest that whilst the parasite does not inhibit all functional responses to LPS-stimulation, such as CD40 expression, *L. mexicana* alone does not effectively activate DCs.

Having demonstrated that the level of DC CCR7 surface expression is modulated by *L. mexicana*, it was next investigated whether the failure of CCR7 up-regulation is due to modulation of CCR7 mRNA expression. Using quantitative PCR, mRNA from DCs exposed to *L. mexicana* was compared with that of unstimulated and LPS activated DCs to determine the relative expression of *ccr7* (Figure 3.2). These data show that the expression of *ccr7* is unaffected after exposure to *L. mexicana*, suggesting that the lack of CCR7 protein surface expression following exposure of DCs to parasites may instead be due to modulation of translation of the mRNA, protein folding, or trafficking of the receptor to the cell membrane.

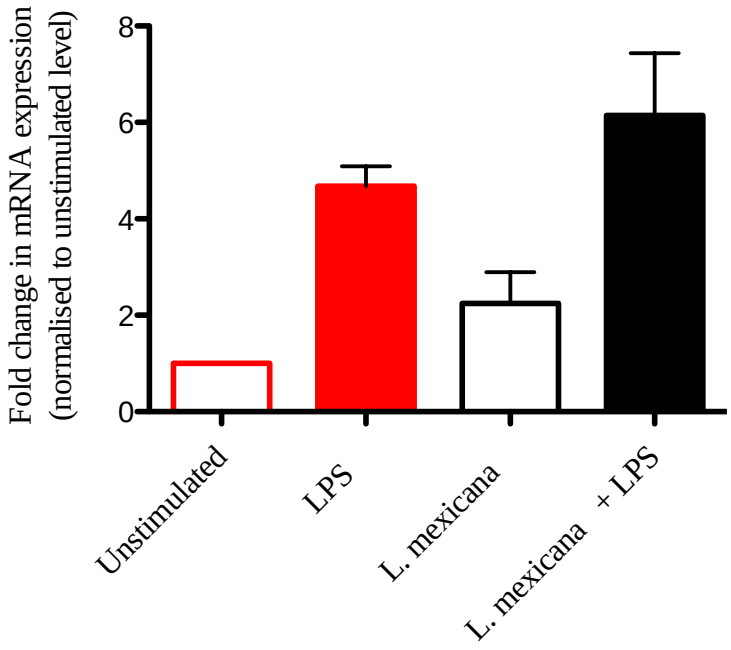


Figure 3.2. Quantitative PCR of CCR7 expression of *Leishmania mexicana* exposed DCs. Bone marrow-derived DCs were infected and stimulated as described in Figure 3.1 and mRNA subsequently extracted. The relative levels of mRNA encoding CCR7 were quantified using real-time PCR. Data show the fold change in mRNA expression as normalised to unstimulated controls and use 18S as a reference gene. Results show the mean ± SEM of 3 experiments each comprising 3 replicate samples. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. No significant (n.s.) differences were identified.

Taken together these data suggest that *L. mexicana* inhibits the LPS-induced upregulation of CCR7 at the cell surface, and that this suppression occurs at the protein level as CCR7 mRNA expression is unaffected by the parasite. Upregulation of the activation marker, CD40, is not induced by *L. mexicana*, although no evidence

suggests that it is suppressed by the parasite. As such, *L. mexicana* appears to avoid a degree of DC activation and may be associated with the suppression of key cellular functions, such as perturbing DC migration.

3.2.2. Exposure to *Leishmania mexicana* inhibits bmDC migration towards CCL19

Having shown that the expression of *ccr7* mRNA is not altered but that the level of CCR7 on the surface of bmDCs is inhibited following exposure to *L. mexicana*, the functional consequence of this was investigated to determine whether the migratory capacity of bmDCs towards CCL19 (CCR7 ligand) was compromised. CCL19 is expressed by the LN and provides a soluble chemokine gradient that stimulates directional migration of CCR7 expressing cells towards the secondary lymphatic tissues. A specialised Ibidi 3D chemotaxis μ-slide (Figure 3.3) was used to develop an assay to image bmDC movement through a collagen matrix and along a CCL19 gradient. This setup provides a 3D structure for cell migration, allowing DCs to migrate via the flowing and squeezing method described by Lammerman *et al* (2008). The slide is designed to form a soluble chemokine gradient across a channel of collagen containing bmDCs (Figure 3.3A). The gel channel can then be imaged using time-lapse microscopy and the migration of cells can be tracked using Volocity software. As shown in Figure 3.3B and C**,** tracks of individual cells can be generated to quantify cell migration over a defined time-course. Subsequently, a number of parameters associated with cell behaviour can be characterised through analysis of cell movement and the type of migratory pattern created (Figure 3.3D)**.** The **displacement** from origin (measured in μm), i.e. the length of the straight path a cell has taken from a point of origin to the end-point, was of particular interest as this

examines how far the cell travels in the direction of the chemokine gradient. The **velocity** of migration (μm/minute) is useful for determining differences in the rapidity of cell motility, and the **meandering index** shows the degree of deviance from a straight pathway, and is a measure of how directional the migration is over the imaging period.

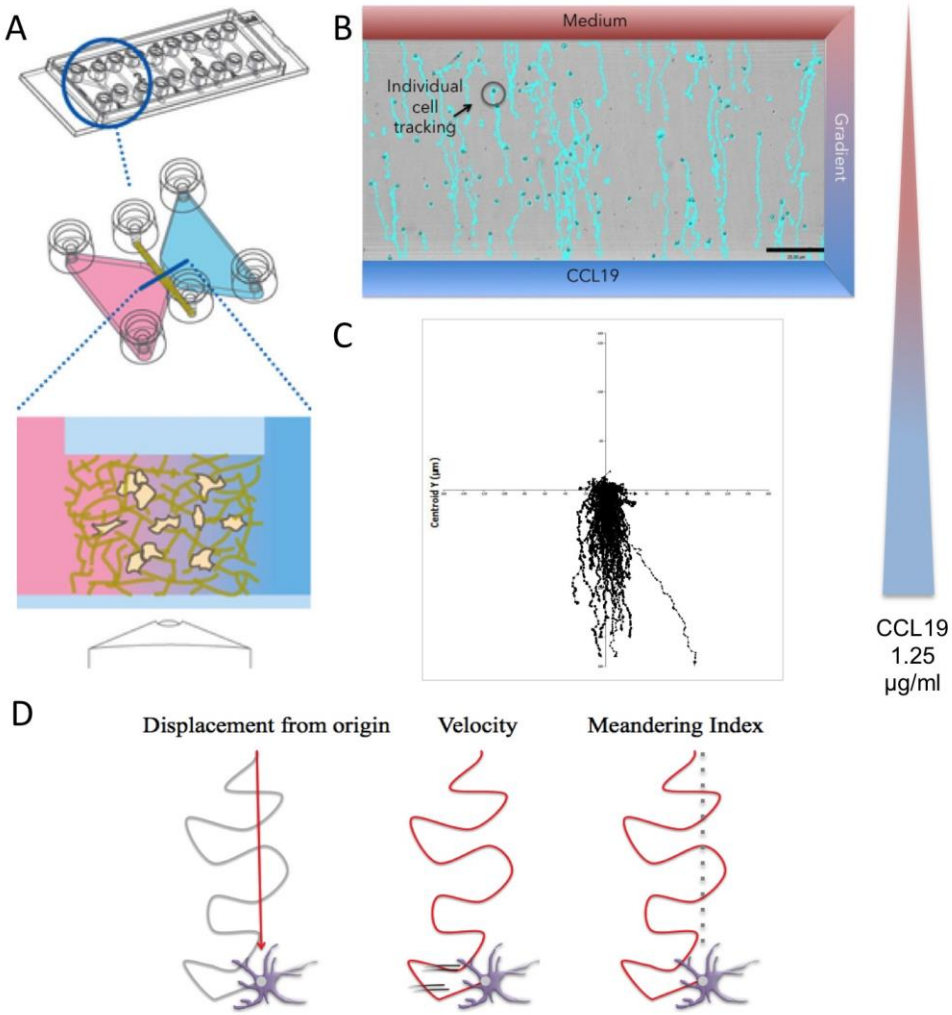


Figure 3.3. Schematic illustrating the Ibidi 3D chemotaxis μ-slide set up and analysis parameters. A) Illustration of the chamber slide showing the chemokine chamber (blue), the RPMI (control) chamber (pink) and the central cell-seeded collagen gel chamber (brown) (figure

adapted from image found at ibidi.com). B) Diagram showing the chemokine gradient across the imaging chamber, including an image of the cells embedded within the collagen matrix and overlaid with the individual cell tracks (turquoise) generated by tracking cells over time in Volocity software. C) Two-dimensional 'flower plot' showing multiple individual tracks of cell migration, overlaid to display a common point of origin. D) A schematic outlining the migration parameters analysed post imaging.

BmDCs were prepared as described above and exposed to parasites and/or stimulated with LPS. Subsequently, cells were embedded within collagen in the imaging chamber of the chemotaxis slide, exposed to a CCL19 gradient, and imaged for a total of 120 minutes. Displacement, velocity and meandering values were calculated for each cell track (over 100 cell tracks per sample), and the mean ± SEM of three replicates for each condition is presented in Figure 3.4 B-D.

As described above, unstimulated bmDCs express low levels of CCR7, and when exposed to a gradient of CCL19, the majority of cells remain relatively localised (Figure 3.4A). Cell tracks of these unstimulated DCs were typically of low displacement (i.e. cells do not move far from a point of origin (Figure 3.4B), low velocity **(**Figure 3.4C), and with a low meandering index (i.e. cells do not show a polarised migration **(**Figure 3.4D). In contrast, LPS activated DCs that express high levels of CCR7, migrate effectively along the CCL19 gradient, forming long and straight cell tracks in the direction of the higher concentrations of chemokine (Figure 3.4A). Thus, DC activation results in high cell displacement and velocity values that

are significantly higher than those of unstimulated DCs, as well as a somewhat increased meandering index (Figure 3.4B-D).

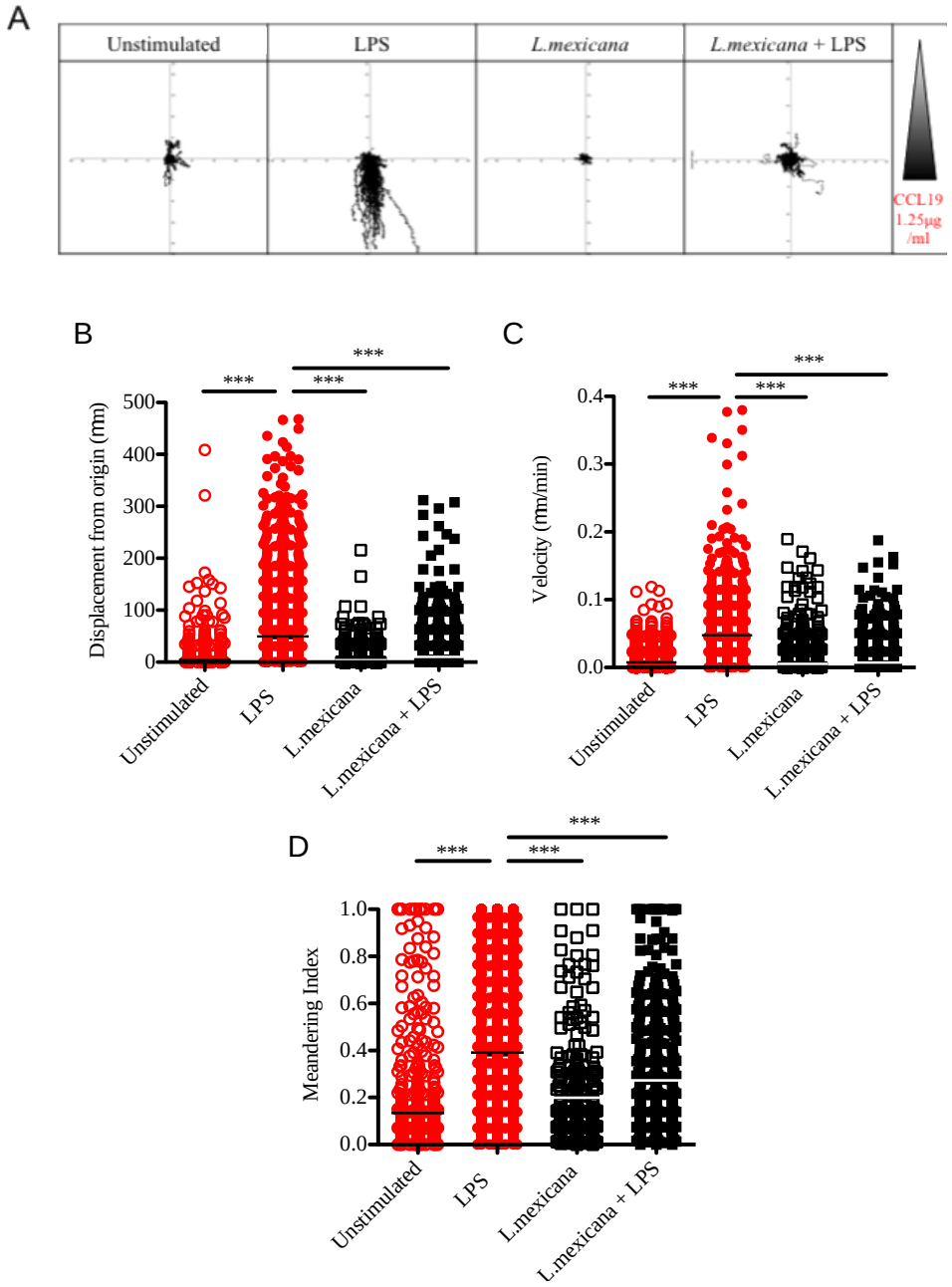


Figure 3.4. *In vitro* chemotaxis of bmDCs is impaired upon exposure to *Leishmania mexicana*. Bone marrow derived DCs were pre-treated as described in Figure 3.1 before seeding in a collagen matrix within a 3D-Chemotaxis µ-slide. A gradient of CCL19 was created across the

imaging chamber, as described in Figure 3.3 and bright-field images were acquired every 2 minutes using an Axiovert inverted microscope with environmental chamber. Cells were tracked as described in Figure 3.3 to create A) flower plots of individual cell tracks for each condition and graphs showing B) displacement of cell from point of origin, C) cell velocity, and D) meandering index of each track. These results show 3 separate experiments and show a data point for each individual cell track (100 cell sample from each independent experiment) as well as the mean (indicated by black line) ± SEM. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. ***=$p<0.001$.

Strikingly, after co-culture with *L. mexicana,* bmDCs failed to migrate along the CCL19 gradient. In fact, the overall migratory profile; meandering index, velocity and displacement did not significantly differ from that of unstimulated cells, as shown in Figure 3.4. Furthermore, bmDCs co-cultured with *L. mexicana* prior to additional LPS stimulation, showed a significantly reduced migratory capacity when compared with bmDCs stimulated with LPS alone and meandering index, velocity and displacement were all significantly reduced. It is striking that even with additional LPS activation, the parasite effectively inhibits DC migration towards CCL19, which would apparently correlate with reduced CCR7 expression by parasite-infected bmDCs as demonstrated under these conditions.

These data suggest that *L. mexicana* promastigotes negatively influence bmDCs migratory capacity, preventing the bmDCs from migrating along a gradient of

CCL19. It would appear that the failure of CCR7 upregulation described in Figure 3.1A may have a detrimental effect on the chemotactic capacity of the cells during *L. mexicana* infection.

3.2.3. *Leishmania mexicana* abrogates dendritic cell migration into lymphatic vessels in BALB/c and C57BL/6 mice

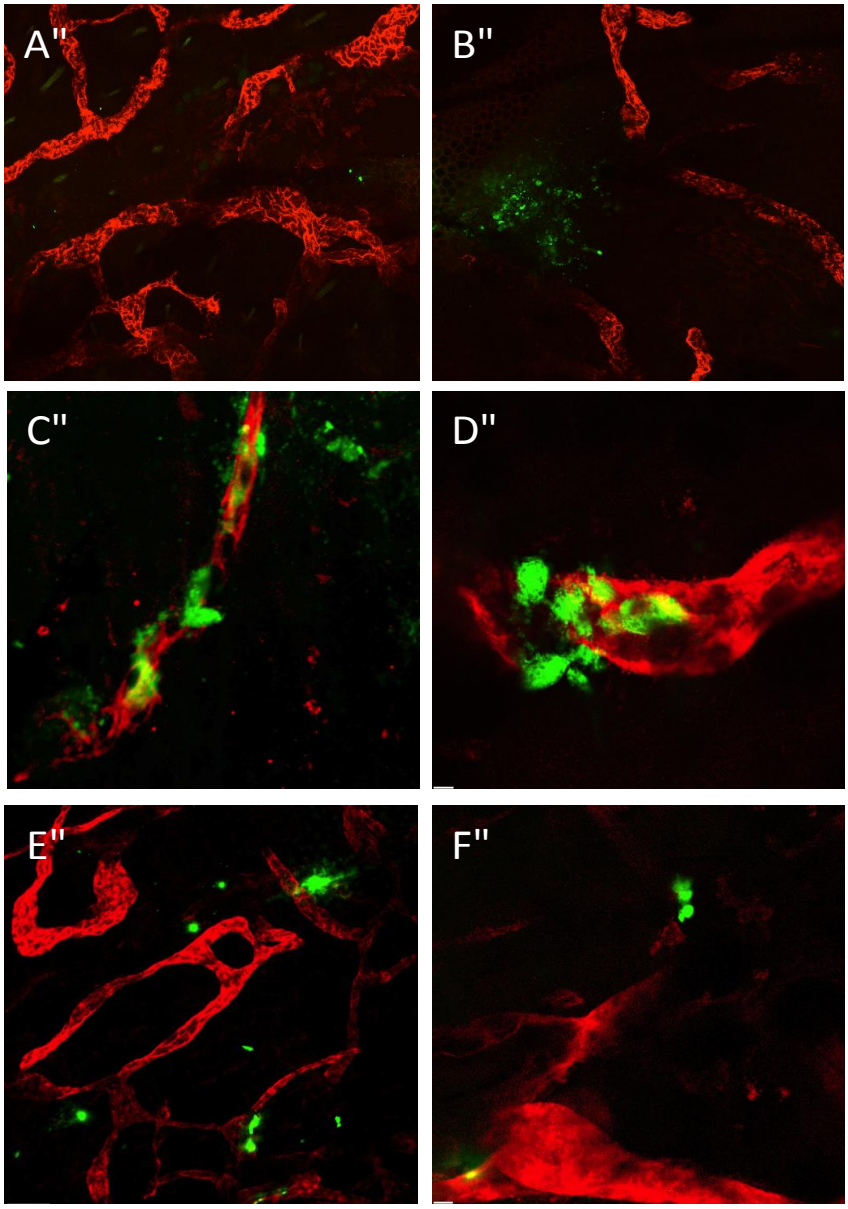
Previous work has suggested that *Leishmania donovani* modulates dendritic cell migration *in vivo* (Ato *et al*, 2002). Having established that CCR7 expression is impaired during *L. mexicana* exposure, and that this appears to have a profound effect on the migratory ability of bmDCs towards CCL19 *in vitro,* it was important to determine whether a similar effect also occurs *in vivo.* Therefore, the next experiments sought to quantify the co-localisation of DCs exposed to *L. mexicana* with lymphatic vessels (LVs) in the skin of either BALB/c or C57BL/6 mice.

BALB/c-derived bmDCs were cultured with promastigotes and/or stimulated with LPS as described above before labelling with CFSE, a fluorescent dye commonly used for cell tracking purposes (Ristevski *et al,* 2003). 10^5 cells were then injected into the naïve ear dermis of BALB/c mice, and the ears were removed 2 hours post transfer. The tissue was fixed, permeabilised and stained with eF-660 conjugated anti-LYVE-1 antibody (red) to reveal LVs. The interaction of the CFSE labelled DCs and the LV network was then imaged using confocal microscopy (Figure 3.5A and B**).**

The degree of colocalisation between green DCs and red LVs in acquired images was quantified by calculating the proportion of green voxels (three-dimensional

pixels) in the entire image that contain above-threshold intensity of red, also known as the overlap co-efficient. This method of analysis controls for the fluctuation of the number of cells between triplicate samples and conditions.

Representative images of LPS-stimulated DCs showed a high degree of migration towards LVs, with clusters of green DCs associated with the vessel (Figure 3.5A and C), as previously described (Tal *et al,* 2011). Conversely, DCs previously exposed to parasites failed to interact with the vessels. (Figure 3.5B and D). Quantification of such images demonstrated that whilst there is a low overlap coefficient between unstimulated DCs and LVs, LPS-activated bmDCs exhibited a high degree of co-localisation with the vessels (Figure 3.5E). Significantly, bmDCs co-cultured with *L. mexicana* promastigotes colocalise with LVs to a lesser degree, with the overlap co-efficient a similar value to that of unstimulated DCs**.** These data demonstrate that exposure to *L. mexicana* negatively effects DC interaction with the LV. These results suggest that the parasite can effectively manipulate DC migration *in vivo.*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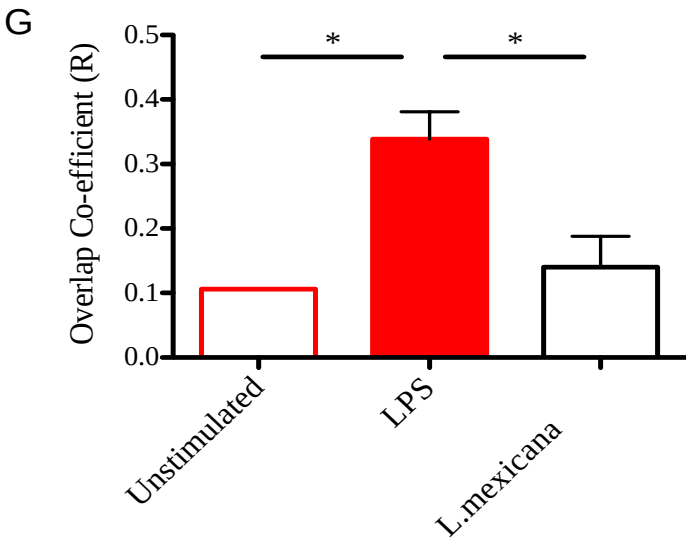
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Unstimulated
LPS
L.mexicana

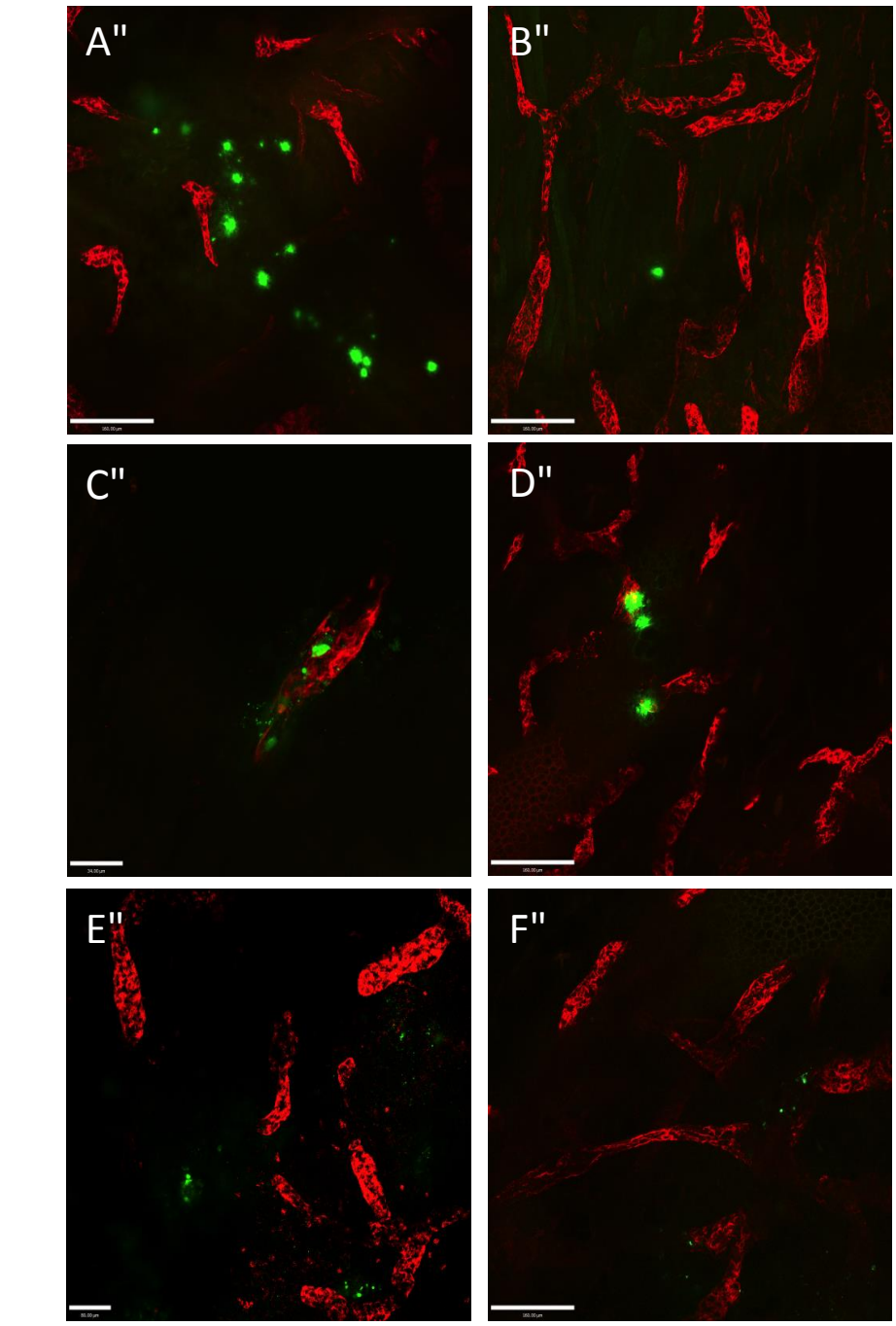
Figure 3.5. BALB/c bmDC interaction with lymphatic vessels *in vivo* is altered following exposure to *Leishmania mexicana*. Bone marrow derived DCs remained unstimulated (A and B), were stimulated with LPS (C and D) or co-cultured with *L. mexicana* (E and F) as described in Figure 3.1. Subsequently, bmDCs were CFSE labeled (green) and 5 x 10^5 cells inoculated into the dermis of a BALB/c ear. 2 hours later, the ear was removed, fixed, permeabilised and stained with eF-660-conjugated anti-LYVE-1 antibody to reveal lymphatic vessels (red) before imaging using confocal microscope (using x 20 (A, B, C and E) and x 40 (D and F) objective lenses). E) **The degree of colocalisation between green bmDCs and red LVs in the images was quantified by calculating the proportion of green voxels (three-dimensional pixels) in the entire field of view, which contained above-threshold intensity of red voxels.** Three-dimensional image stacks were analysed to quantify bmDC colocalisation with lymphatic vessels (expressed as the overlap coefficient) using Volocity software. Images shown are representative of 3 mice per group and the data presented in (G) show the mean ± SEM of three images for each described condition. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. *=$p<0.05$.

The same experimental parameters were studied in C57BL/6 mice as it is accepted that genetic background can influence disease outcome to *Leishmania* infection (reviewed McMahon-Pratt and Alexander 2004). In the case of *L. major* BALB/c mice are inherently susceptible to infection and C57BL/6 mice are resistant, whereas infection with *L. mexicana* is non-healing and persistent in both BALB/c and

C57BL/6 mice. It was therefore necessary to investigate whether the development of chronic infection, induced by *L. mexicana* in both strains, could be attributed to a failure in DC interaction with the LV.

The experiment was repeated as above but using C57BL/6 bmDCs and recipients and ear tissue was imaged at 2 h and 24 h post cell transfer (Figure 3.6). Interestingly, an identical pattern of DC-LV co-localisation was observed in C57BL/6 mice to that of the BALB/c mouse. Again, images showed that DCs previously exposed to parasites failed to migrate towards the LV at both 2 h and 24 h post transfer and quantification of 3D images revealed a low overlap co-efficient between *L. mexicana* exposed DCs and the LV network relative to that of LPS-stimulated DCs (Figure 3.6E). These results suggest that DC-LV interaction is extremely limited in C57BL/6 mice as well as in BALB/c mice, and that this deficiency in migration is maintained from 2 h up to 24 h after cell transfer.

In summary, the data presented so far suggest that the migration of DCs towards CCL19 and the lymphatic vessel is down-regulated if DCs are exposed to *L. mexicana*. These results show that whilst CCR7-expressing LPS-stimulated DCs will readily migrate towards and intravasate LVs, exposure of DCs to *L. mexicana* fails to initiate this DC-LV contact. Therefore these *in vivo* observations directly correlate with the *in vitro* responsiveness of treated DCs to CCL19 demonstrated in Figure 3.4 These data suggest that the failure of CCR7 up-regulation on the surface of bmDCs in the presence of *L. mexicana* has an inhibitory effect on the ability of bmDCs to colocalise with or intravasate the CCL19 expressing, lymphatic vessels *in vivo*.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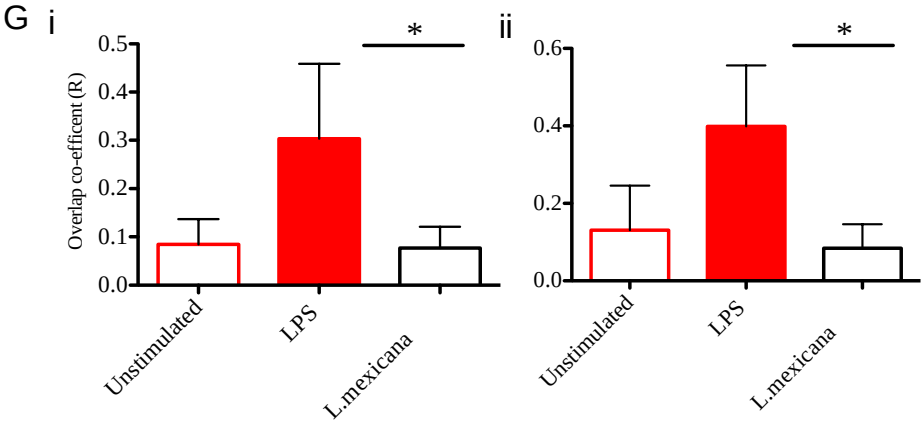
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Unstimulated
LPS
L.mexicana

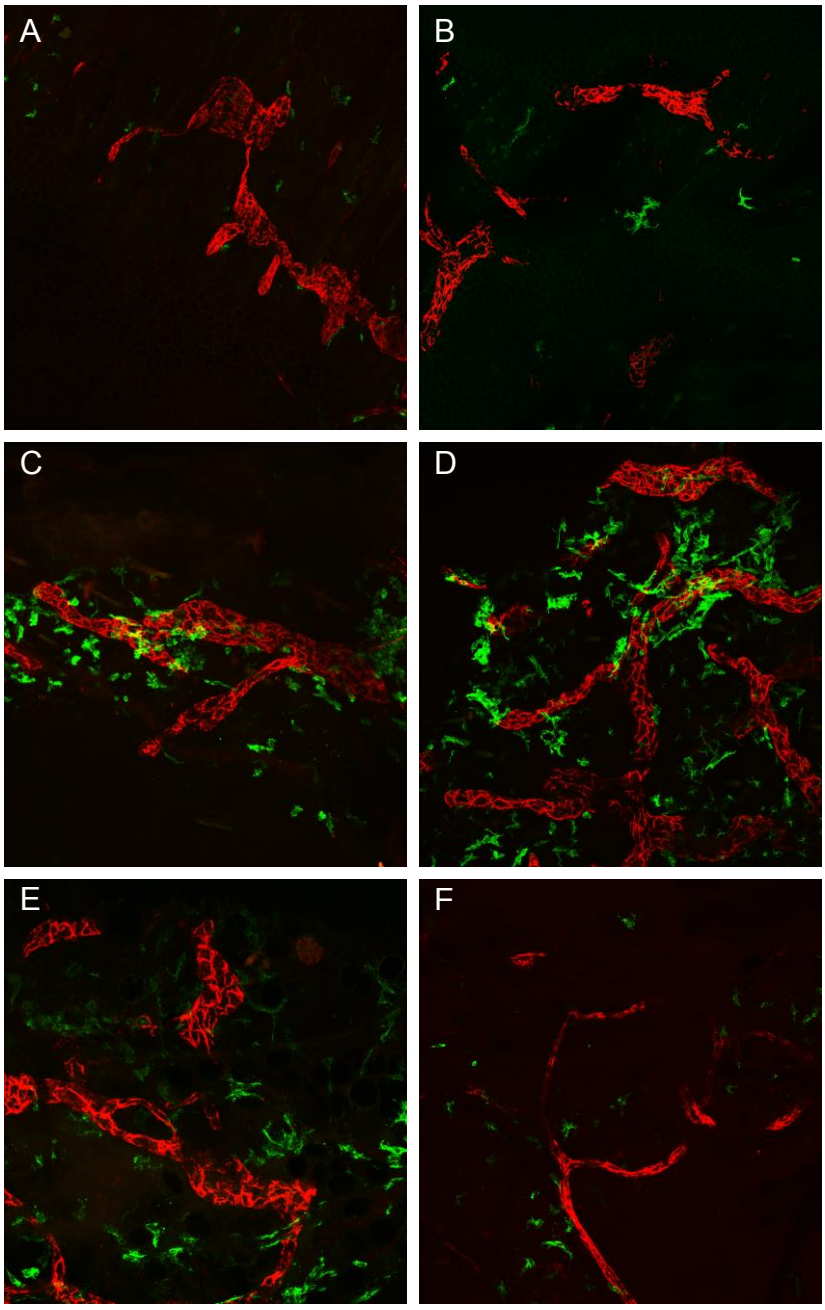
Figure 3.6. C57BL/6 bmDC interaction with lymphatic vessels *in vivo* is altered upon pre-exposure to *Leishmania mexicana*. BmDCs were CFSE labeled (green) and 5 x 10^5 cells inoculated into the dermis of C57BL/6. At 2 hours and 24 h post injection, ears were removed, fixed, permeabilised and stained with eF660-conjugated anti-LYVE-1 antibody to reveal lymphatic vessels (red). A) 2 h and B) 24 h unstimulated, C) 2 h and D) 24 h post LPS-stimulation E) and F) post *L. mexicana* infection. Tissue was imaged by confocal microscopy. G) Quantification of bmDC colocalisation with lymphatic vessels using overlap co-efficient at i) 2 h and ii) 24 h post injection. All analysis was carried out using Volocity software and the proportion of red-green overlap in each of the images was determined as previously described in **Figure 3.5**. Images shown are representative of 3 images and 3 mice per group. Scale Bar = 160 μm. The data presented in E) shows the mean ± SEM of three images for each described condition. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. *=$p<0.05$.

3.2.4. Migration of MHC Class II-expressing cells is perturbed by *Leishmania mexicana* infection *in vivo*

Having shown an inhibitory effect exerted by *L. mexicana* on the migratory capacity of bmDCs exposed to parasites *in vitro*, it was important to understand the response of resident cells *in vivo* during *L. mexicana* infection. Whilst a number of approaches have been use to visualise DCs *in vivo*, including CD11c-YFP reporter mice, these animals were not available within the time-frame of this project. Previous reports

have used whole-mount staining of skin tissue to identify MHC class-II-expressing cells (Tal *et al,* 2011). Whilst staining for MHC class-II does not specifically identify DCs, because other cell types such as macrophages express this marker, at the early time-points after initial infection the majority of MHC class II^+ cells are DCs (Ng *et al,* 2008).

Infection was initiated by inoculating 10^6 *L. mexicana* promastigotes into the ear dermis of BALB/c mice. After 2 or 24 hours, ears were removed, fixed and permeabilised and prepared for immunofluorescent staining, using a FITC-conjugated anti-MHC class II^+ (green) to reveal endogenous antigen-presenting cells, and anti-LYVE-1 eF660 to stain LV. Confocal microscopy was used to visualise the cellular interaction of the MHC Class II^+ cells with the LV through a 3-dimensional volume of the infection site (Figure 3.7).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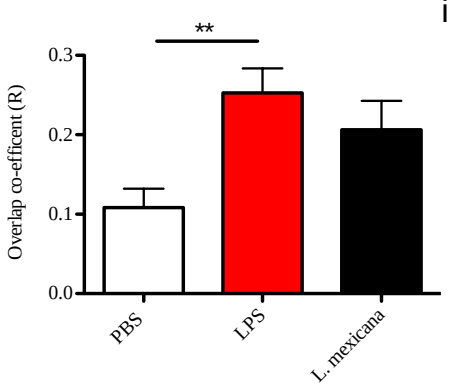
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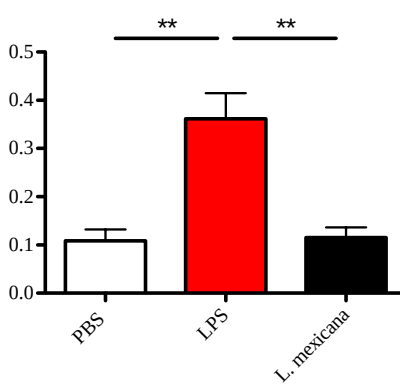
LPS
L. mexicana

ii)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PBS
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L. mexicana

Figure 3.7. Interactions between endogenous MHC Class II+ cells and lymphatic vessels are modulated by *Leishmania mexicana in vivo.* Representative confocal images of whole mount BALB/c mouse ear preparations stained with anti LYVE-1 (red) and anti-MHC Class II^+ (green). Images represent 2 h (A, C & E) or 24 h (B, D & F) post injection of either PBS (A & B) 100 ng LPS (C & D) or $1x10^6$ *L. mexicana* promastigotes (E & F) into the dermis. (G) Quantification of confocal images, showing the overlap coefficient at i) 2 h and ii) 24 h post infection, were calculated by measuring green objects (>1μm) detected in lymphatic stained regions (red) using Volocity software, as previously described in **Figure 3.5**. Images are representative of three experiments and represent 3 mice per group. The graph (G) Results show the mean ± SEM of 3 images for each described condition. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. **=$p<0.01$ and ***=$p<0.001$.

Representative images of PBS-injected tissue suggested that few MHC Class II expressing cells localise to the LV network and of the cells detected, there is little interaction between the cells and LV at either time point (Figure 3.7A and B). Conversely, in LPS-stimulated tissue, a larger MHC Class II-expressing population was observed and there was a high degree of cell contact with LVs at both 2h (Figure 3.7C) and 24h post LPS stimulation (Figure 3.7D). However it was specifically at 24h post LPS treatment that accumulated MHC Class II expressing cells formed clusters that were closely associated with the LV (Figure 3.7D), as previously described (Tal *et al,* 2011). Whilst *L. mexicana* infection was associated with an

increase in MHC Class II-expressing cells relative to PBS-injected animals, largely these cells failed to show any interaction with the LV (Figure 3.7E and F). Quantification of images (Figure 3.7Gi and ii) demonstrated that MHC class II-expressing cells in naïve tissue had a low overlap coefficient i.e. little interaction with LVs. Conversely, tissue injected with LPS showed a greater degree of colocalisation between MHC Class II^+ cells and LVs, with a higher overlap coefficient. Importantly, although *L. mexicana* infection appears to stimulate a level of recruitment of MHC class II-expressing cells, these cells demonstrate a failure to colocalise with the LVs at both 2 hours and 24 hours post-infection. Most notably, at 24 hours after infection the overlap coefficient of MHC class II^+ cells with LVs was similar to the level of naïve tissue and significantly less than that of the LPS stimulated tissue (Figure 3.7Gii).

These data suggest that the migratory ability of resident MHC class II^+ cells and interaction with lymphatic vessels is greatly reduced during *L. mexicana* infection, most significantly at 24h post infection. In agreement with the work presented in Figure 3.6, these data suggest that migratory functionality of antigen-presenting MHC-Class II expressing cells – most likely tissue resident DCs (Tal *et al,* 2011) – is affected *in vivo* during infection as well as in an *in vitro* system.

3.2.5. Dendritic cell migration to the draining lymph node is significantly reduced following *Leishmania mexicana* infection.

The experiments above suggest that *L. mexicana* reduces the level of functional CCR7 on the surface of DCs, abrogating cell migration towards CCL19 *in vitro* and

migration is also impaired *in vivo*, with reduced colocalisation of DCs with the lymphatic vessels in two model systems. Activated bmDCs migrate from the lumen of the LV to the paracortex T cell region of the draining lymph node (LN), where antigen-presenting DCs will present peptide in the context of MHC Class II to naïve T cells leading to the generation of a specific adaptive response (Schumann *et al,* 2010). Recent work has suggested that reduced migration of DCs during *Leishmania* infection will mean reduced DC-T cell interactions and a failure to develop a protective Th1 response (Ato *et al,* 2006; Petritus *et al*, 2012). *L major* has been shown to modulate Langerhans cell (LC) migration to the dLN in C57BL/6 mice (Steigerwald and Moll, 2005; Sato *et al.*, 2000) and Ato *et al* (2006) have demonstrated that *L. donovani* can inhibit DC migration to the spleen. Therefore, it was next investigated if the failure of DC migration towards LV - associated with L. mexicana - may have an influence on accumulation of DCs in the draining lymph node. A combination of confocal imaging and flow cytometry was used to quantify the absolute number of DCs present in the draining lymph node post adoptive transfer. BmDCs were stimulated with LPS or co-cultured with *L. mexicana* (as described above), prior to CFSE labelling and transfer of 10^6 cells into the footpad of BALB/c recipients. Twenty four hours post transfer, the draining lymph nodes were removed and either prepared for tissue processing, immunofluorescent staining and imaging; or for flow cytometry.

To generate quantification of DC accumulation in an entire lymph node using an imaging-based method, serial cryosections of frozen tissue were prepared and stained with anti-B220-Pacific Blue (to identify B cell follicles - blue) and anti-CD11c-APC (to identify DCs – red). Images of serial sections were acquired using a stitch-and-

tile method to construct an image of each LN slice and then reconstructed into a 3D image using the counterstains as “landmarks”. Representative 3D reconstruction imaging of LN isolated from recipients of LPS-stimulated and *L. mexicana*-exposed DCs can be seen in Figure 3.8A and B respectively. Importantly, using analysis software, the number of green, cell-sized objects detected in the entire LN could be quantified and correlated with the results obtained using flow cytometry.

Using this approach, few events were detected in the draining lymph node 24 h post transfer of unstimulated DCs, implying that resting DCs do not readily migrate to the draining LN and supporting earlier data. In contrast, LPS-activated DCs successfully accumulated in lymph nodes, with high numbers of transferred $CFSE^{+}$ DCs readily identified in the LN tissue. Notably, *L. mexicana* exposed DCs failed to migrate to the dLN, that is to say that few DCs were imaged in the tissue, with absolute cell numbers similar to that of the unstimulated sample (Figure 3.8). To ensure that quantification of 3D reconstruction imaging was truly representative, flow cytometry was used to identify the absolute number of CD11c-expressing CFSE labelled DCs in the lymph node. The results were in concordance with the imaging data (Figure 3.8C and D), with low numbers of unstimulated cells in the draining LN, a significantly larger population of LPS-stimulated cells, and a small population of *L. mexicana* exposed DCs detected in the tissue. However, further to this DCs were exposed to *L. mexicana* and also stimulated with LPS and it was found that, even upon LPS stimulation, few DCs were detected.

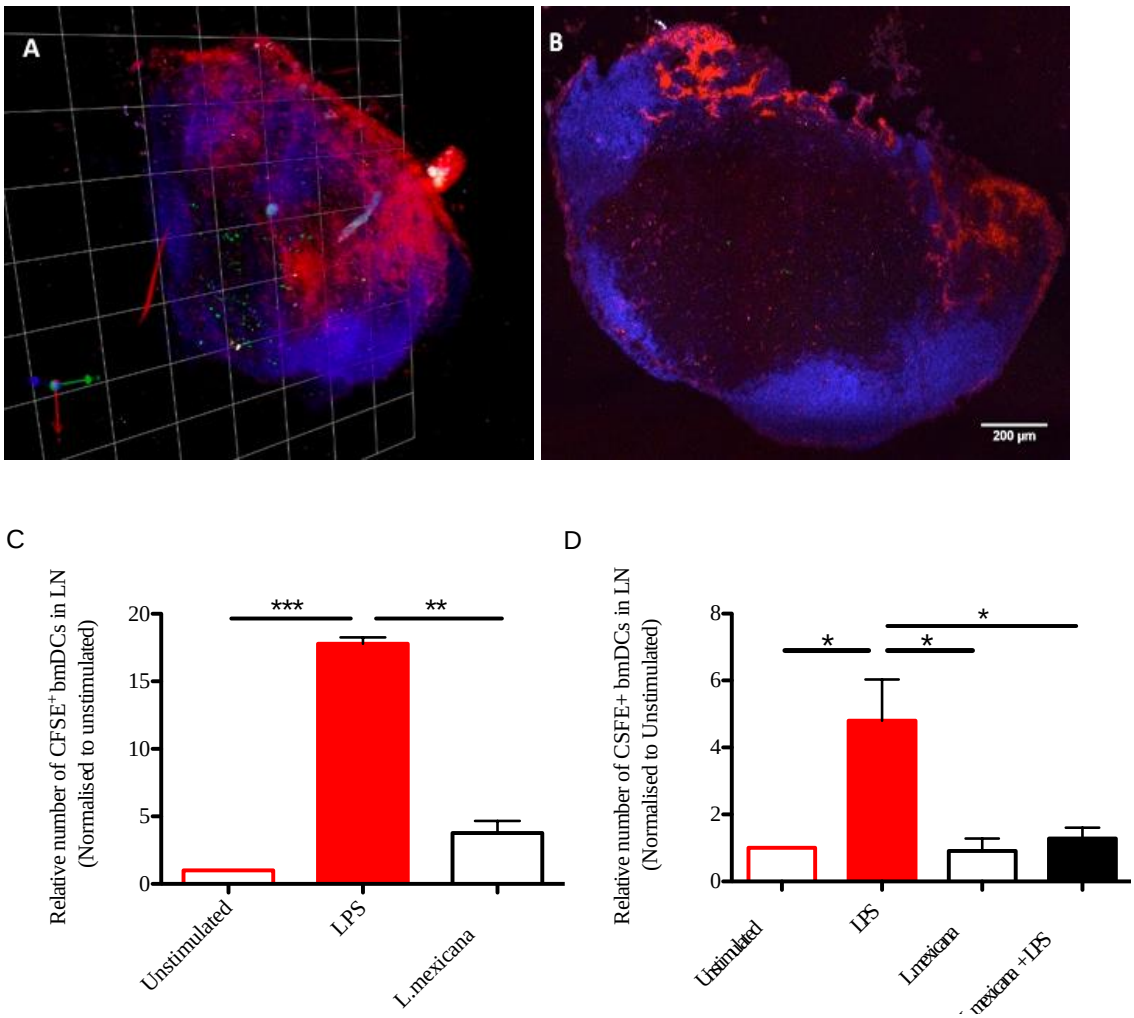


Figure 3.8. BmDCs pre-exposed to *Leishmania mexicana* exhibit impaired migration to the draining lymph node. BmDCs were prepared as in **Figure 3.5** and 10^6 cells injected into the footpads of BALB/c mice. At 24 h post-injection, popliteal LN were removed and either prepared for serial cryosectioning or flow cytometry. Imaging of whole popliteal LNs was carried out acquiring a stitch-and-tile confocal image of each serial section and then reconstructing images into a 3-dimensional composition in Volocity. Images were acquired of whole LNs containing CFSE-labeled A) LPS-stimulated or B) *L. mexicana*-exposed DCs and C) the number of green objects quantified. For

comparison, D) the relative number of $CFSE^+$ DCs in the popliteal LN were quantified using flow cytometry. Images are representative of three experiments and represent 3 mice per group. The graphs show the mean ± SEM of the three experiments comprising 3 replicate samples. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. *=p<0.05 **=p<0.01 ***=p<0.001.

Together these data support that *L. mexicana* does not trigger DC migration to the draining LN and that even upon additional LPS stimulation, exposure to *L. mexicana* renders the DCs unable to migrate to the draining LN. c

3.2.6. Exposure of antigen-loaded DCs to *Leishmania mexicana* influences activation of T cells responses.

As discussed above, the generation of a T cell response is dependent on the presentation of antigen by DCs to naïve T cells. Having shown that exposure to *L. mexicana* will inhibit DC migration to the draining lymph node, it was next necessary to determine the effect this will have on T cell activation and expansion. The rarity of antigen, and the environment in which DCs present antigen to the naive T cell can influence the robustness of a T cell response (Obst et al, 2005; Schumman, 2010; Itano and Jenkins, 2003). Therefore, it was next investigated what effect this failure in DC migration might have on the activation and subsequent proliferation of T cells in the draining LN.

$3x10^6$ CFSE-labeled OVA-specific T cells from OT-II donor mice were transferred into C57BL/6 recipient mice. Congenic Ly5.1 DCs were pulsed with OVA and pre-exposed to *L. mexicana* and/or stimulated with LPS, then transferred into the footpad

of the recipient mouse. After 72 h hours, the popliteal lymph node was removed and flow cytometry was used to analyse the proliferation of the CFSE labeled T cells. Dilution of the CFSE fluorescence intensity correlates to cell division cycles, thus allowing quantification of the number of rounds of T cell division. Figure 3.9A shows the gating strategy used to quantify the proportion of OVA-specific T cells. Vα2 and Vβ5 TCR-specific antibodies were used to identify the transferred T cell population in the popliteal lymph node. In recipient mice that received un-pulsed DCs, a small number of OVA-specific OT-II T cells were present in the LN and there were no statistical differences between mice that received LPS-stimulated DCs or *L. mexicana* exposed DCs (Figure 3.9B). Conversely, in recipients of DCs that were loaded with OVA peptide, there was an increase in both the percentage and absolute number of OVA-specific OT-II T cells in the draining LN (Figure 3.9Bi and ii). Although the frequency of OVA-specific T cells was slightly lower in recipients of OVA-pulsed DCs that had been exposed to *L. mexicana* (relative to LPS-stimulated DCs), these differences did not achieve significance. However, analysis of CFSE intensity revealed that the proportion of OVA-specific OT-II T cells entering cell division was significantly influenced by DC exposure to *L. mexicana.*

Thus, whilst T cells recognising OVA presented by LPS-activated DCs underwent up to 6 or 7 rounds of division (Figure 3.9C), T cells stimulated by *L. mexicana* exposed, OVA-pulsed DCs appeared to undergo fewer rounds of division (Figure 3.9C). Indeed, quantification of the absolute number of dividing OVA-specific T cells in the dLN (Figure 3.9D) demonstrated that exposure of OVA presenting DCs to *L. mexicana* significantly impaired T cell division.

The preliminary data generated from this pilot experiment suggests that exposure of DCs to *L. mexicana* may result in an alteration of T cell division phenotype, further supporting the findings above that migration of these DCs to the LN is impaired following uptake of parasites.

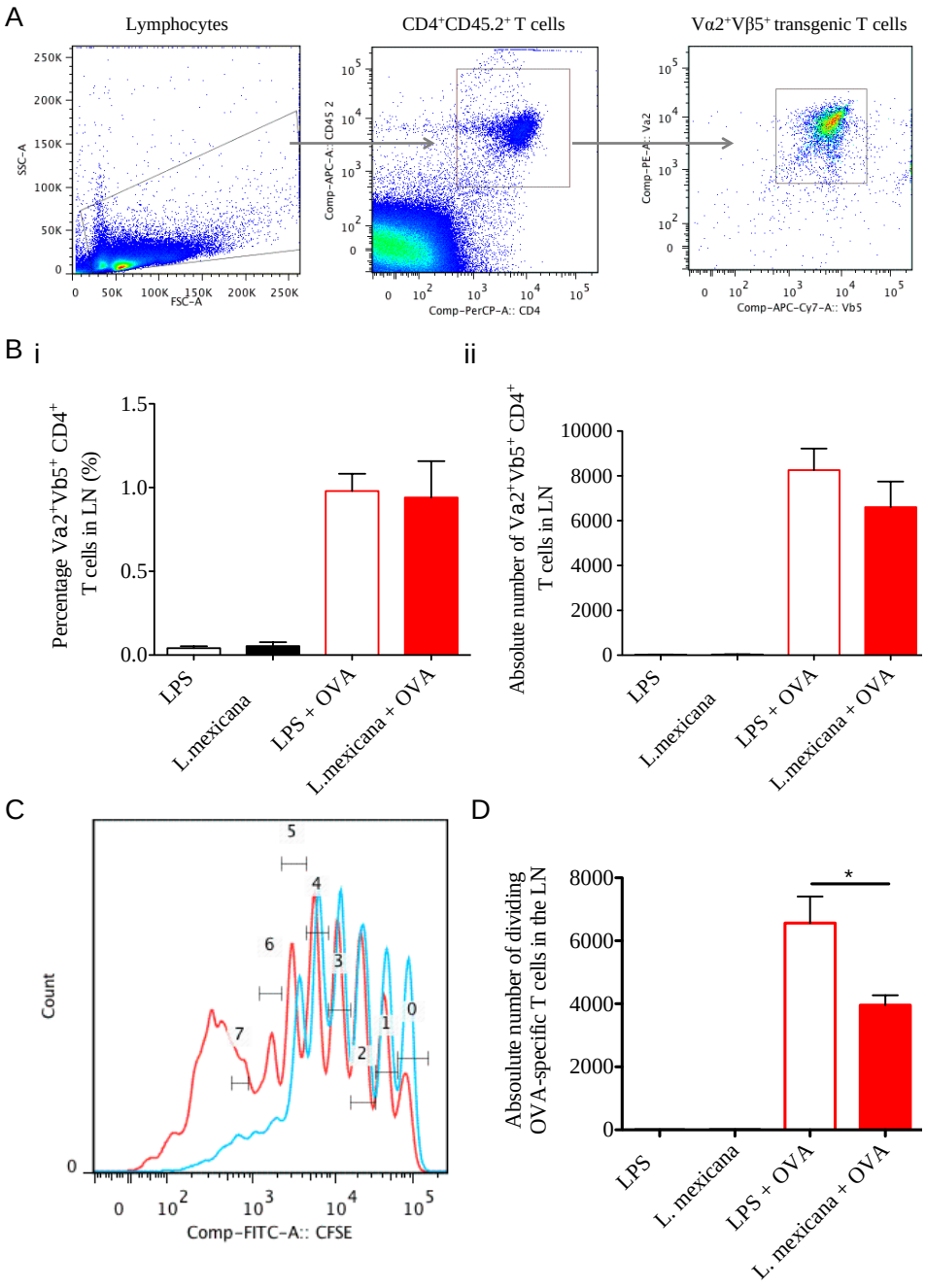


Figure 3.9. Exposure to *Leishmania mexicana* reduces DC capacity to induce T cell proliferation. 3x10^6 CFSE-labeled OT-II were transferred

into C57BL/6 recipient mice, which then received Ly5.1 DCs pre-treated as in **Figure 3.1** and OVA-pulsed (1 mg/ml), or remained un-pulsed. After 72 h, the popliteal LN was isolated and cells prepared for flow cytometry. The gating strategy to identify lymphocytes, $CD4^{+}V\alpha2^{+}V\beta5^{+}$ T cells is illustrated in (A). The i) percentage and ii) absolute number of $CD4^{+}V\alpha2^{+}V\beta5^{+}$ T cells in the LN were quantified in (B). (C) is a representative plot showing the CFSE intensity of $CD4^{+}V\alpha2^{+}V\beta5^{+}$ lymphocytes in recipients of OVA-pulsed LPS-stimulated (red line) of L. mexicana co-cultured (blue line) DCs, where 0 is the undivided population (E) shows the absolute number of dividing OVA- specific $CD4^{+}V\alpha2^{+}V\beta5^{+}$ T cells in the LN. Results show the mean ± SEM from one experiment with 3 mice per group. Statistical analysis performed using 1-way ANOVA and Bonferroni post-test. **=$p<0.01$. Statistical differences identified between the absolute number of dividing *L. mexicana* exposed and LPS stimulated OVA pulsed $V\alpha2^{+}V\beta5^{+}$ T cells.

3.3. Discussion

Dendritic cell migration in both steady-state and in inflammation is important for initiation regulation of adaptive immune response. The skin provides a natural barrier to infection for the host, but is commonly breached and thus this organ houses a complex network of dendritic cells that can rapidly migrate throughout the tissue to process, transport and present self or foreign antigen to the draining LN (Meglio *et al,* 2011). Specifically, it has been shown that during *Leishmania* infection DC subsets received by the draining lymph nodes, are primarily skin derived DCs, such as Langerhans cells (LCs), dermal DCs (DDCs) (Kautz-Neu *et al,* 2011), and monocyte derived DCs (moDCs) (Petritus *et al,* 2012). In non-*Leishmania* models, migration of these DCs is largely dependent upon CCR7 expression, a chemokine receptor expressed on the surface of dendritic cells and T cells, allowing DCs to migrate into the lymphatic vessels and towards lymph nodes (Ohl *et al*, 2004). CCR7 expression is classically upregulated upon interaction with a pathogen (Forster *et al*, 1997). The migration of mature DCs will then occur along a soluble gradient of CCL19 and CCL21 largely expressed by LN stromal cells, T cells and other DCs and toward the LV (reviewed by Randolph *et al,* 2005; Tal *et al*, 2011) and the draining lymph node (Luther *et al*, 2002). Interestingly, a significant number of infectious agents, most notably viruses, have been shown to downregulate or fail to upregulate DC CCR7 expression and inhibit migration in response to CCL19, including HSV-1 (Prechtel *et al,* 2005), HCMV (Varani *et al,* 2005) and Vaccinia Virus (Humrich *et al*, 2007) as part of their immune evasion mechanisms. Furthermore, Ato *et al* (2002) have demonstrated that DC CCR7 surface expression is inhibited during *L. donovani*

infection, and that treatment with CCR7-expressing DCs curtailed parasite replication.

With this in mind, it was hypothesised that, as in the case of *L. donovani,* the chronicity of *L. mexicana* infection could be a result of impaired DC CCR7 expression following exposure to parasites. Therefore, it was investigated whether *in vitro* exposure to *L. mexicana* modulated CCR7 expression on bmDCs. In line with the findings of Ato *et al* (2002), it was demonstrated that exposure to *L. mexicana* promastigotes *in vitro* resulted in a failure in the upregulation of CCR7 surface expression. Interestingly, whilst the CCR7 surface expression is modulated by the parasites, qPCR results suggested that CCR7 mRNA is effectively expressed after *L. mexicana* exposure, and that the presence of CCR7 on the surface of bmDCs is instead modulated at a protein level, or perhaps during trafficking to the cell surface. Indeed, it has been suggested that *Leishmania* can effectively degrade surface receptors during intracellular trafficking. Parasite derived virulence factors, such as cysteine protease (CP) have been implicated in this process and MHC Class II molecules are functionally destroyed by these proteases, which are actively secreted from the parasitophorous vacuole (De Souza Leao *et al,* 1995). Further to this CPs have been identified in the extracellular matrix (Ilg et al 1994*,* Mottram *et al,* 2000) and could possibly have a role in receptor cleavage at the cell membrane.

Having shown that CCR7 surface expression is significantly lowered after exposure to *L. mexicana* it was important to determine whether this could have a functional impact on the migratory capacity of the DCs towards CCL19. These findings demonstrated that bmDCs exposed to *L. mexicana* failed to migrate along a CCL19 gradient. With the use of a μ-slide chemotaxis assay, it was demonstrated that the

velocity and displacement of bmDCs exposed to parasites were similar to that of unstimulated cells and significantly lower than that of LPS stimulated DCs. Moreover, even with the addition of LPS, exposure to parasites effectively inhibited DC migration towards CCL19. These data suggest that the failure of bmDC to up-regulate CCR7 expression after *L. mexicana* exposure has a detrimental effect on the chemotactic capacity of the cells. It should be noted that CCL19 is not the only chemokine required for the functional migration of CCR7 expressing cells. However, the soluble nature of CCL19 allowed for the investigation of how DCs will interpret a gradient of chemoattractant (Schumman *et al,* 2010). CCL21 also plays an important role in the docking and intravasation of DCs with the LV (Tal *et al,* 2011; Lammerman *et al,* 2008), and thus, further investigation is required to determine whether CCL21 driven DC migration is similarly affected during infection.

Nonetheless, the findings presented here are remarkably similar to the evidence presented by Ato *et al*, (2002) in which *L. donovani* has been shown to down-regulate the migration of DCs within the secondary lymphatic organs and that this is directly because of non-responsiveness to CCL19/CCL21 gradients.

In addition, it may also be considered that following internalisation by the host macrophage *L. donovani* is capable of sequestering F-actin to the parasitophorous vacuole, and in doing so protects against endosome-fusion and parasite killing (Holm *et al,* 2001). F-actin is a known component required for the migration and adhesion of DCs (Lammerman *et al,* 2008), and whilst there has been no evidence to suggest that macrophage migration is affected by this process, it could be possible that *L. mexicana* may also dysregulate F-actin function and in this way cause a generalised inhibition of DC migratory capacity.

In light of the findings presented in Figure 3.2.2, it was hypothesised that the chemotaxis of bmDCs towards LV may be disrupted during *L. mexicana* infection *in vivo*. Recent advances in imaging techniques have allowed the direct visualisation of DC chemotaxis in both steady state and during inflammation (Sixt *et al*, 2002; Sen *et al*, 2010). Visualisation of DC migration *in vivo* is ideal for understanding the complex interplay between the parasite and the host. To date, the migration of DCs from the peripheral site of *L. mexicana* infection in the skin to the lymphatic vessels, and lymph node is yet to been defined. Therefore, confocal imaging was used to visualise the interaction between adoptively transferred bmDCs and LVs in both BALB/c and C57BL/6 mice.

It was demonstrated that, similarly to unstimulated bmDCs, DCs co-cultured with *L. mexicana* promastigotes failed to interact with LVs. In fact, *L. mexicana*-exposed DCs had significantly less contact than LPS-stimulated DCs in both BALB/c and C57BL/6 mice. Numerous studies have shown that genetic background will affect the progression of *Leishmania* infection. Classically, BALB/c mice are susceptible to infection and C57BL/6 mice are resistant to *L. major* infection, where as infection with *L. mexicana* causes chronic disease in both mouse strains (Alexander *et al,* 1985; Rosas *et al,* 2005). Recently, neutrophils have been shown to play a major role in facilitating infection and inhibiting influx of moDCs and a Th1 response in C57BL/6 mice (Hurrell et al 2015). *L. mexicana* parasitises neutrophils silently both in terms of inflammatory cytokine and chemokine secretion unlike *L. major* (Charmoy et al 2007 and 2010) that induces CCL3 that causes an influx of inflammatory monocytes. The present study does not preclude such a mechanism but indicates a further mechanism that can influence DC migration into and out of the

site of infection and draining lymph nodes, namely a failure of *L. mexicana* to upregulate DC CCR7 expression, and significantly *L. mexicana* exposure did not stimulate bmDCs migration towards LV in either BALB/c or C57BL/6 mice. It could therefore be hypothesised, that reduced DC-LV interaction contributes to the chronicity of *L. mexicana* infection in both mouse strains. Taken together this evidence supports that the reduced responsiveness of *L. mexicana*-exposed DCs to CCL19 *in vitro* appears to directly correlate with failure of DC migration towards LV *in vivo*, and the indication is that these observations may be attributable to a parasite-mediated inhibition of CCR7 expression by bmDC. Interestingly, the sandfly saliva product maxadilin, that has been associated with promoting initial *Leishmania* infection, also down regulates CCR7 expression (Wheat *et al*, 2008) indicating multiple pathways for immune evasion.

Transfer of *L. mexicana*-exposed DCs into syngeneic hosts is a useful tool to determine if *in vitro* exposure to the parasite has an effect on the migratory capacity of DCs. However, quantifying the migration of endogenous DCs during infection would be the optimal method for delineating this complex set of interactions *in vivo*. For example, multiphoton microscopy has been utilised to image real-time migration of CD11c-specific GFP expressing reporter mice and Ng *et al* (2008) have used this approach to visualise the dynamics of dDC parasite uptake and retrieval of antigen (Tal *et al,* 2011; Ng *et al,* 2008). However, due to the time constraints of the present project it was not possible to use these methods. Therefore, mice were directly infected with *L. mexicana* and the infected tissue was removed at multiple time points post-infection for whole mount staining of endogenous antigen presenting cells and the LV. Anti-MHC Class II antibodies were chosen to stain antigen-

presenting cells and, whilst this method does not allow for specific labeling of DCs, it has been demonstrated that at this time following initiation of inflammation it is likely that the majority of MHC Class II cells will be DCs (Tal et al 2011). Such an approach revealed that, in support of previous DC transfer studies (Figure 3.5 and Figure 3.6) during live *L. mexicana* infection endogenous antigen-presenting cells fail to interact with LVs.

The evidenced failure of dendritic cell migration and altered chemokine receptor expression during *Leishmania* infection will affect the essential DC-T cell interactions required for the generation of a healing T cell response. Therefore, how DC chemotaxis is modulated during infection is key in understanding how the parasite can manipulate the host immune response. Recently, it has been shown that impaired DC migration during *Leishmania* infection results in the failure of functional DC migration to the draining LN (Petritus *et al,* 2012; Hurrell *et al,* 2015). These workers have shown that moDC migration in chronic *L. mexicana* infection is impaired. During infection, monocyte recruitment to the site of infection is reduced, recruited moDCs produce less iNOS and fewer moDCs are found in the draining LN after infection. Further to this, it has been demonstrated that *L. major* can inhibit Langerhans cell migration through the manipulation of CCR2 expression (Steigerwald and Moll, 2005) and *L. donovani* can modulate CCR7 expression (Ato *et al,* 2002). These factors all contribute to impaired DC migration to the draining LN and result in reduced DC-T cell interactions.

Consequently, it was considered whether the reduced interaction of *L. mexicana* exposed DCs with LVs would result in a failure of DC accumulation in the draining LN. The number of adoptively transferred bmDCs in the draining lymph node 18 h

post cell transfer was quantified by confocal imaging of serial sections of a whole LN. The images were then combined to form a 3D reconstruction of the whole LN, from which DC quantification could be achieved. Importantly, using this approach, it was also possible to determine the position of the DCs within the LN.

Certainly, it has been demonstrated that activated bmDCs will migrate from the lumen of the LV to the paracortex T cell region of the draining LN, where DCs will present antigen in the context of MHC Class II to naïve T cells initiating an adaptive immune response (Schumann *et al,* 2010). Importantly, during *L. donovani* infection Ato *et al* (2006) used CCL19/CCL21 deficient mice to demonstrate that reduced DC migration to the T cell region of the spleen, and thus fewer DC -T cell interactions, exacerbates disease.

Even with additional LPS stimulation, significantly fewer *L. mexicana*-exposed bmDCs successfully reached the draining lymph node in comparison with LPS-stimulated DCs. In line with the findings demonstrated by Petritus *et al*, (2012) these data suggest that not only does *L. mexicana* not induce DC migration to the draining LN, but also exposure to *L. mexicana* will render DCs unable to migrate to the draining LN, actively suppresses the chemotaxis of these cells. Further to this, the apparent lack of DC migration to the draining LN observed upon exposure to *L. mexicana* may influence T cell activation and proliferation, and could potentially have a negative impact on parasite clearance.

To test this, CFSE labeled transgenic OT-II T cells, with OVA specific TCR were used to quantify the extent of OVA antigen presentation by DCs pre-exposed to *L. mexicana*. Whilst there was no significant difference in the percentage of CFSE

labeled T cells after transfer (Figure 3.9Bii) with LPS stimulated DCs or *L. mexicana* exposed DCs, it was found that the number of T cells entering into division is significantly reduced when presenting DCs have been exposed to *L. mexicana* (Figure 3.9D). It should be considered that even subtle differences in T cell proliferation and expansion profiles can have a substantial impact on the development of T cell immunity (Smith *et al,* 2002). Importantly, the paucity of migrating DCs in the LN may influence the DC-T cell interaction. These data suggest that fewer T cells enter into division upon transfer of antigen on *L. mexicana*-exposed DCs and of those T cells that do start to divide, they may undergo fewer rounds of division. Therefore, it is possible that the rarity of antigen loaded DCs results in fewer DC-T cell interactions and therefore has a negative impact on the number of T cells dividing. Indeed, it has been shown that the rarity of antigen and antigen presenting DCs will influence the adaptive immune response (Obst *et al,* 2005). These workers show that unlike $CD8^+$ T cells, $CD4^+$ T cells proliferate in accordance with the amount of antigen peptide they encounter and if that antigen persists during the expansion phase of proliferation. This could have significant implications for *L. mexicana* infection, where my work has shown that DC migration to the secondary lymphoid organs is impaired. This impaired migration will provide only rare opportunities for MHC Class II peptide complex-TCR interactions and, whilst we have shown that this may not initially affect the activation and proliferation of OT-II T cells, it may be important for the latter stages of T cell immunity development.

In summary the work in this chapter examined the impact of *Leishmania mexicana* on DC migration. DCs co-cultured with *L. mexicana* promastigotes fail to

express CCR7, are refractory to LPS-stimulation and show reduced migration towards CCL19 *in vitro*. Furthermore, *in vivo*, DCs exposed to *L. mexicana* fail to interact with lymphatic vessels and thus DC migration to the draining lymph node is significantly reduced. Moreover, it is demonstrated that this may have implications for the generation of an adaptive protective immune response.

These data demonstrate that *L. mexicana* can impair immune activation and that perturbation of DC migration plays a key role in this process. Parasite-derived virulence factors, such as cysteine proteases, have been implicated in the inhibition of intracellular signaling (Cameron *et al,* 2005) and degradation of MHC Class II molecules during intracellular trafficking (de Souza *et al,* 1995). Therefore, it is possible that these proteases could target DC chemokine receptor surface expression during infection and as such, the work in Chapter 5, proceeded to examine this hypothesis. However, before that work commenced, I next wanted to explore the hypothesis that exogenous stimulation of DC migration away from the site of infection may enhance the development of a "healing" Th1 type immune response. Through therapeutic administration of a drug that has shown to be effective in stimulating CCR7 expression on the surface of DCs, it may be possible to overcome the parasite-mediated downregulation of the receptor, and thus may consequently trigger effective DC migration to the draining LN following *L. mexicana* infection.

Chapter 4. An evaluation of the therapeutic potential of Resiquimod-mediated DC stimulation during *L. mexicana* infection

4.1. Introduction

The evidence presented in Chapter 3 demonstrates that the reduced expression of CCR7 by DC following exposure to *L. mexicana* may be associated with a failure of DC migration in *vitro* and *in vivo*. It was demonstrated that DC migration towards LV and their subsequent accumulation in the draining LN was significantly impaired and thus, it has been hypothesised that the failure of DC migration during *L. mexicana* infection will contribute to the development of chronic infection.

R-848 (know as Resiquimod) is a member of the imidazoquinoline family of synthetic molecules, which are used primarily as immune stimulators and modifiers. Imiquimod (Aldara^{TM}) is the most commonly researched form of the imidazoquinalones, and is a licensed drug commonly formulated for topical treatment of viral skin disorders, such as HPV (Edwards *et al,* 1998), and some types of skin cancer (Geisse *et al,* 2004). However, recent studies have demonstrated that R-848 can activate TLR7 and TLR8 signalling pathways (Ahonen *et al,* 1999; Jurk *et al,* 2002) and previous work has demonstrated the drug's effectiveness in stimulating activation of skin resident Langerhans cells (Burns *et al,* 2000).

Importantly, a number of studies have demonstrated that imidazoquinolines can efficiently stimulate immune cell migration. Initially, a study by Suzuki *et al* (2000), investigated the use of topically administered imiquimod in the modulation of DC migration. The authors demonstrated that after imiquimod treatment, Langerhans cells changed morphology, migrated away from the skin tissue (also demonstrated by Tripp *et al,* 2008), and into the draining LN. Similarly, Yrlid *et al* (2006) have shown that, following oral administration of R-848, intestinal DCs migrate to the mesenteric LNs. Together these studies provide convincing evidence that R-848 treatment

efficiently stimulates DC migration away from the treated tissue and towards the draining LN.

Given its ability to activate TLR7/8 and stimulate DC migration, R-848 has been applied as an adjuvant during antigen challenge and evidence suggests that it can enhance Th1 type antibody production (Vasilikos *et al,* 2000). Further to this, R-848 (Resiquimod) has been trialled for use as an adjuvant for vaccination using a *Leishmania* antigen (Zhang and Matlashewki, 2010). Specifically, Zhang and Matlashewski (2010) demonstrated that whilst subcutaneous vaccination with *L. major* antigen alone does not confer protection against subsequent infection, co-administration with R-848 resulted in a significant increase in IFN-γ production and IL-4 and IL-10 cytokine production was significantly reduced. The upregulation of Th1 cytokines and inhibition of Th2 type adaptive immunity suggests that R-848 is successful in stimulating the development of a Th1 type healing response, and importantly a degree a protective immunity was observed upon challenge infection. These reports suggest that R-848 can enhance the development of protective immunity against *L. major* infection.

Therefore, it was hypothesised that the administration of R-848, which stimulates DC migration to the draining LN, may reverse the impaired DC chemotaxis observed during *L. mexicana* infection, and in this way, stimulation of DC migration could contribute to the resolution of chronic infection. Thus, the aim of this chapter was to investigate whether stimulation of DC migration during initial *L. mexicana* infection could alter the outcome of disease and associated immune response *in vivo*.

4.2. Results

4.2.1. R-848 stimulation of bmDCs triggers upregulation of CD40 and CCR7.

With an overall aim to restore DC migration following *Leishmania* infection, it was first important to confirm that R-848 stimulation would successfully activate bmDCs and enhance CCR7 expression *in vitro*. To confirm activation of bmDCs following R-848 treatment, the expression of CD40, a DC activation marker expressed upon activation of TLR signaling pathways (Hernandez *et al,* 2007), and CCR7 were quantified. Using flow cytometry, $CD11c^+$ bmDCs were identified and the proportion of CD40-expressing cells was assessed by determining the proportion of cells showing staining above that of isotype controls (Figure 4.1A). As expected, stimulation with either LPS or with 10 μg/ml R-848 (Torii *et al,* 2008; Ahonen *et al,* 1999) resulted in a significant increase in the percentage of CD40 expressing cells relative to unstimulated DCs. As anticipated, LPS activation also significantly increased the proportion of CCR7-expressing bmDCs (Figure 4.1B) when compared with unstimulated DCs. Equally, these data confirm that R-848 treatment successfully induced CCR7 expression and thus caused a significant increase in the proportion of $CCR7^+$ DCs. Thus, R-848 can be used to activate DCs and these cells are likely to have the capacity to migrate towards CCR7 ligands.

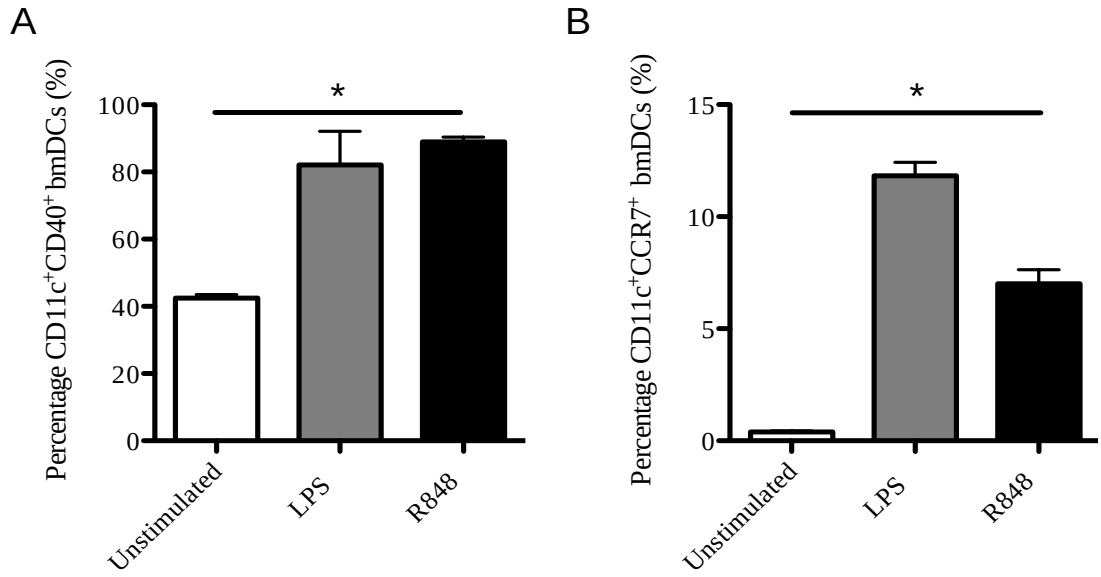


Figure 4.1. Expression of CD40 and CCR7 by bmDCs is increased by R-848 stimulation. Bone marrow-derived DCs were either unstimulated or stimulated with LPS (1 μg/ml) or R-848 (10 μg/ml) for 24 hours prior to flow cytometry analysis to determine the proportion of $CD11c^+$-gated cells expressing A) CD40 or B) CCR7. **Results show the mean ± SEM of 3 experiments each comprising 3 replicate samples.** Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. *=$p \leq 0.05$. Significant differences between unstimulated and LPS/unstimulated and R-848 in both A) and B).

4.2.2. Administration of R-848 effectively enhances DC migration *in vivo*

Having established the functionality of R-848 *in vitro*, it was necessary to perform a pilot experiment to determine the optimal dosage for *in vivo* use of R-848 in stimulating DC migration from the footpad to the draining lymph node. Therefore, BALB/c mice were injected into the footpad with a range of R-848 doses (or DMSO

diluent alone) and the total number of $CD11c^+$ DCs in the draining popliteal lymph node calculated 18 hours post-injection (Figure 4.2).

These results demonstrate that, whilst DC migration was negligible following administration of DMSO alone, a dose of 25 ng of R-848 resulted in a notable increase in the total number of DCs detected in the LN (Figure 4.2). Whilst, it is possible that these results were also associated with improved retention of DC in the LN, these data do indicate that this dosage can effectively modulate DC function in vivo. As such, it was determined that the lowest effective concentration of 25 ng was a suitable dosage for future work.

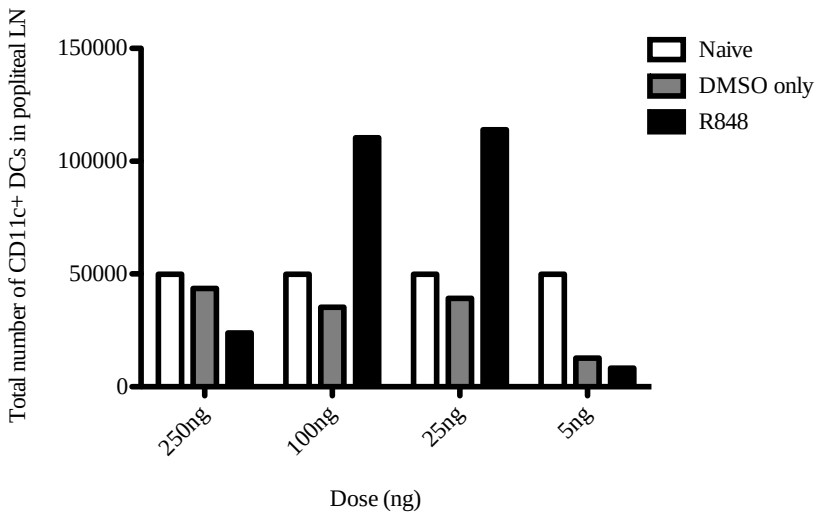


Figure 4.2. Administration of R-848 stimulates DC accumulation in the draining LN. BALB/c mice were s.c. injected into the hind-footpad with either DMSO or the indicated doses of R-848. Flow cytometry was performed on whole lymph node homogenate and the absolute number of $CD11c^+$ cells calculated. Results show a pilot experiment, with each bar representing one individual mouse.

4.2.3. Therapeutic administration of R-848 following *Leishmania mexicana* infection enhances lesion development and Th1 responses.

Having determined that R-848 effectively stimulates CCR7 expression *in vitro* and also drives DC migration *in vivo*, it was hypothesised that R-848 could be used as a trigger for DC migration during *L. mexicana* infection. As demonstrated in Chapter 3, the parasite can significantly impair DC migration *in vitro* and *in vivo*. Therefore, could the additional stimulation provided by R-848 possibly overcome this inhibition of DC motility? As an initial experiment, it was investigated whether a single dose of R-848, administered post-*L. mexicana* infection could alter the progression of disease.

BALB/c mice were injected into the hind footpad with $2.5x10^6$ *L. mexicana* metacyclic promastigotes. As demonstrated in Chapter 3, and previously by other workers (Tripp *et al,* 2008; Tal *et al,* 2011; Petritus *et al,* 2012), activated DCs will migrate towards and into the LVs between 18 h and 48 h post-activation. Thus, it was reasoned that DCs, previously shown to have impaired migratory capacity (Chapter 3), should be stimulated 18 h after infection to potentially stimulate optimal DC migration. As such, 18 hours post-infection, R-848 (25 ng) was injected into the same footpad. The infected footpads were measured at weekly intervals, and the progression of disease was determined by calculating lesion size over time. The results for three experimental repeats are presented in Figure 4.3-Figure 4.6, with each experiment including 5 mice in each group.

Figure 4.3A and B suggest that lesion growth is significantly increased in R-848-treated animals compared with control infected mice injected with PBS alone. However, a third repeat of the experiment (Figure 4.3C**)** failed to demonstrate a clear difference in lesion development, although a small increase in lesion size was evident

between weeks 5 and 6 post-infection in R-848-treated mice. Taken together, these data suggest that there is a trend towards an increase in lesion development when R-848 is administered shortly after *L. mexicana* infection.

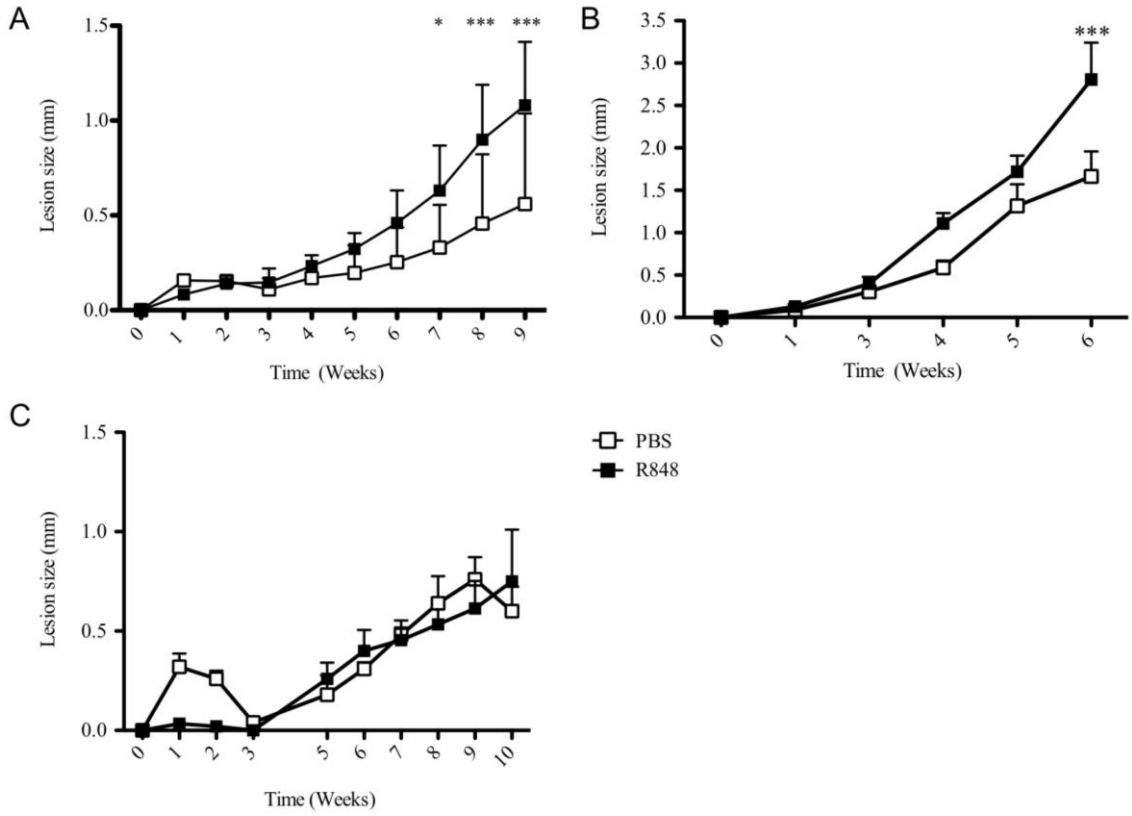


Figure 4.3. R-848 treatment exacerbates lesion growth during *Leishmania mexicana* infection. BALB/c mice were infected in the hind footpads with $2.5x10^6$ *L. mexicana* metacyclic promastigotes and subsequently treated in the same footpad with R-848 (25 ng; filled symbols) or PBS alone (empty symbols) 18 h after infection. The lesions were measured using spring-loaded calipers at weekly intervals over the course of infection. Lesion size was calculated by subtracting the size of the contralateral footpad from that of the infected footpad (mm). Each

panel represents data from a separate experiment and shows the mean ± SEM of a group of 5 mice. Statistical analysis performed using 2-way ANOVA with Bonferroni post-test. *=p≤0.05, **=p≤0.01, ***=p≤0.001. Statistical differences between R-848 and PBS control groups.

Further to this, limiting dilution assays were performed on the excised footpad lesions in Experiment 1, 2 and 3 (Figure 4.4A, B, C) to determine parasite burden in the footpad. The results showed no discernable difference in parasite numbers after treatment with R-848.

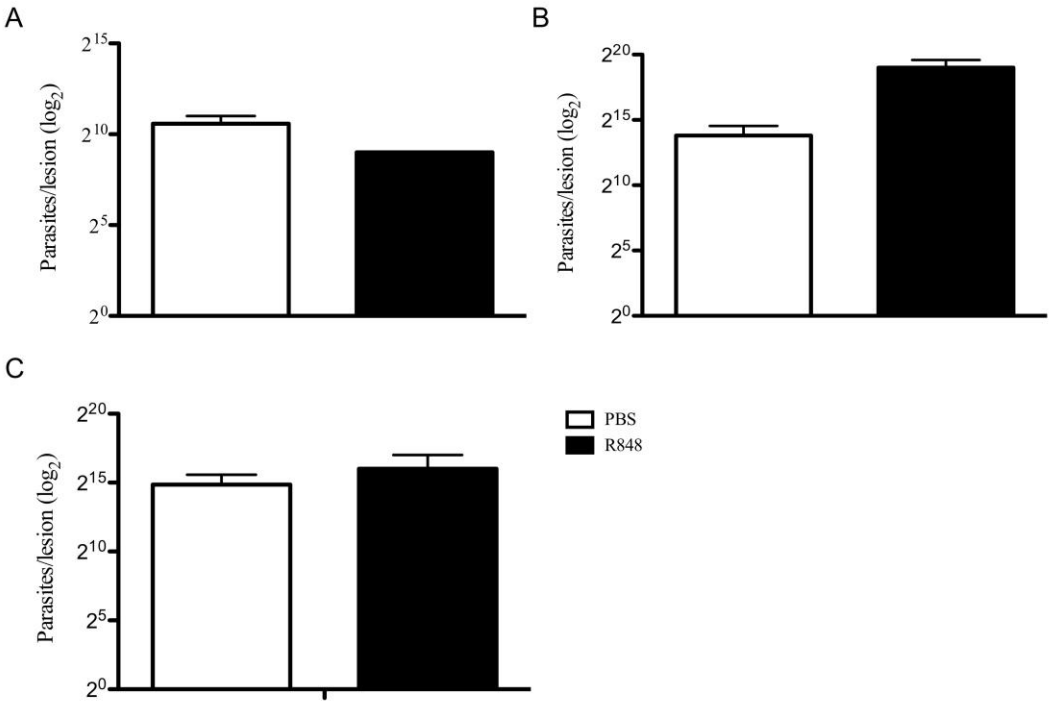


Figure 4.4. Parasite enumeration in *Leishmania mexicana*-infected footpads following R-848 treatment. Mice were infected and treated as described in **Figure 4.3**. After sacrifice of the mice, the footpad lesion was excised, disrupted and the resulting cell suspension was tested by

limiting dilution assay to determine the mean parasite burden. Each panel represents data from a separate experiment and shows the mean ± SEM of 3 mice per group. Statistical analysis performed using Mann-Whitney U test. No statistical differences between R-848 and PBS control groups.

Haematoxylin and eosin staining (H&E) of excised lesions was used to assess the level of cellular infiltrate at the infection site following R-848 treatment. Images suggest that there was extensive cellular infiltrate in both R-848-treated and control, PBS injected mice following *L. mexicana* infection (Figure 4.5). Analysis of the images suggested that there was no noteworthy difference in lesion structure and, because of the non-specific nature of H&E staining, it was not possible to distinguish if there was any difference in various immune cell population sizes.

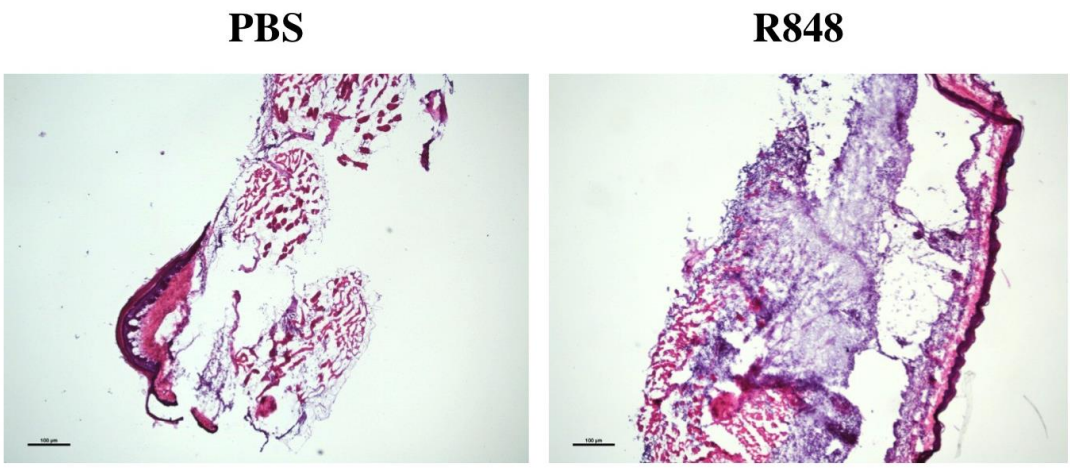


Figure 4.5. Histological staining of *Leishmania mexicana*-infected footpads following R-848 treatment. Mice were infected and treated as described in **Figure 4.3**. After sacrifice of the mice, the excised footpad lesions were frozen at -80 °C in OCT freezing medium and prepared for cryostat sectioning. 8 mm thick sections were stained with haematoxylin

and eosin stain (described fully in methods) and imaged using an upright Nikon (x 20 objective lens). Scale bar = 100 μm.

Having shown that R-848 treatment leads to enhanced lesion development following *L. mexicana* infection, it was next pertinent to determine how treatment may affect the generation of adaptive immunity. Initially, blood serum was tested to determine the level of parasite-specific IgG1 and IgG2a by calculating the reciprocal end-point dilution using an antibody ELISA. Again, there was some variability between replicate experiments. However, in both Experiment 1 and 3 (although not in Experiment 2), mice treated with R-848 soon after infection with *L. mexicana*, showed significantly higher titres of *Leishmania*-specific IgG2a than PBS-treated infected mice (Figure 4.6). There were no significant differences in the titres of parasite-specific IgG1 between R-848-treated and PBS-injected control mice.

Together, these data suggest that there is a trend towards enhanced IgG2a production associated with therapeutic treatment with R-848 soon after *Leishmania* infection. However, it was next necessary to acertain whether the observed increase in IgG2a, is assocated with enhanced Th1 type cytokine expression. Therefore, cytokine production by *Leishmania*-specific T cells isolated from spleens of infected mice treated with R-848 was quantified. There were no significant differences in either IFN-γ or IL-4 production associated with R-848 treatment in either Experiment 1 or 2, although in these experiments only very low levels of parasite-specific cytokines were produced (data not shown).

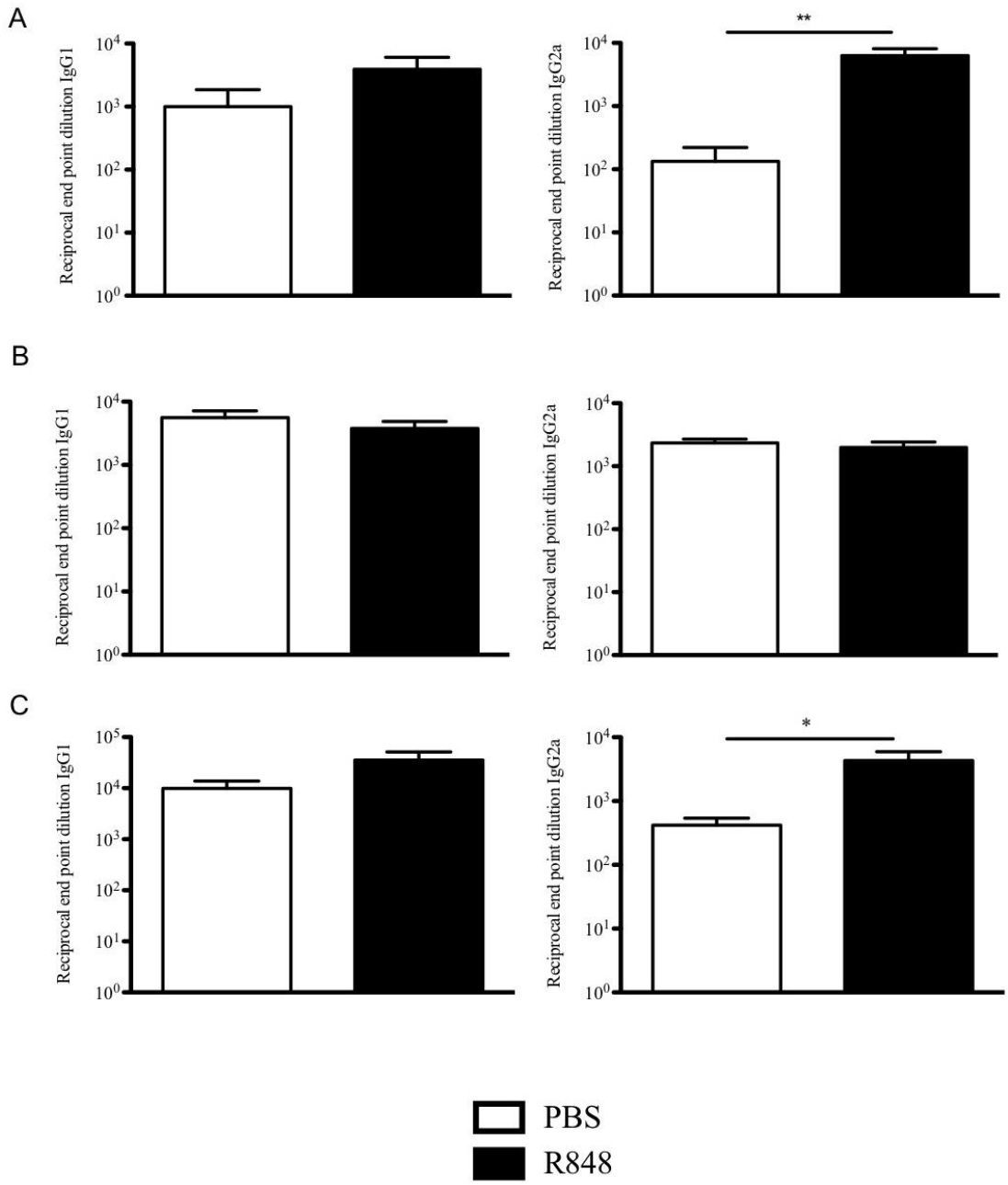


Figure 4.6. R-848 treatment significantly enhances *Leishmania mexicana*-specific IgG2a antibody production.

Mice were infected and treated as in **Figure 4.3** and at the end of the experiment the levels of parasite-specific IgG1 and IgG2a were established using antibody ELISAs. Each row represents data from a separate experiment and shows the mean reciprocal end-point dilution of

5 mice per group ± SEM of 5 mice. Statistical analysis performed using an Unpaired T- test. * =p≤0.05 , ** =p≤0.01.

Originally, it was hypothesised that a single dose of R-848 would drive early DC migration, and susbsequntly this would then enhance the development of a healing Th1 type adaptive response. The results presented above suggested that a single treatment with 25 ng R-848 is moderately effective at enhancing production of parasite-specific IgG2a, indicating a bias towards a Th1 response. However, it is not clear that this is a direct result of R-848 specific enhancement of DC migration. In addition, lesion development is significantly enhanced following R-848 treatment (in 2 of 3 experiments) and though this is not indicative of increased parasite burden and could be the result of enhanced inflammation, this type of adverse reaction following treatment suggests that a single dosage of R-848 following *Leishmania* infection is not a viable treatment option.

4.2.4. Repeated treatment with R-848 over the early stages of *Leishmania mexicana* infection enhances lesion development

As mentioned previously, it has been established that skin resident DCs will migrate to the LV within the 18 h to 48 h window (Randolph *et al,* 2005). However, recent work by Tripp *et al* (2008) has demonstrated that different subsets of DCs, i.e. dermal DC and Langerin-positive Langerhans cells, intravasate the LV at different rates following inflammation.

In the previous experiment (Section 4.2.3), it was considered that a single dose of R-848 at 18 h following infection was the optimum time to stimulate migratory DCs.

Indeed, this work demonstrated that this R-848 dosage generated moderately enhanced IgG2a responses to the parasite, although parasite burdens were not uniformly reduced by treatment. However, in light of the findings by Tripp *et al* (2008) it may be the case that a single dosage at this early time point is not sufficient to drive migration of all DC subsets that may contribute to the development of the adaptive response. Therefore, it was reasoned that by administering multiple R-848 doses over the first weeks of infection, the sustained migration of skin resident DC subsets might be stimulated, overcoming the *L. mexicana* specific defect and inducing immunity.

Consequently, the basic experimental approach described in **Section 4.2.3.** was utilised, with the addition that treatment with R-848 was injected into the infected footpad at multiple time-points following infection. Mice were treated with R-848 at regular intervals (0 h, 1 week and 2 weeks post-infection) in an attempt to overcome the suppressed DC migration throughout this early stage of infection. Similar to the pattern observed in mice treated with a single dose of R-848, mice treated with multiple doses of R-848 following infection went on to develop significantly larger lesions than control mice (Figure 4.7).

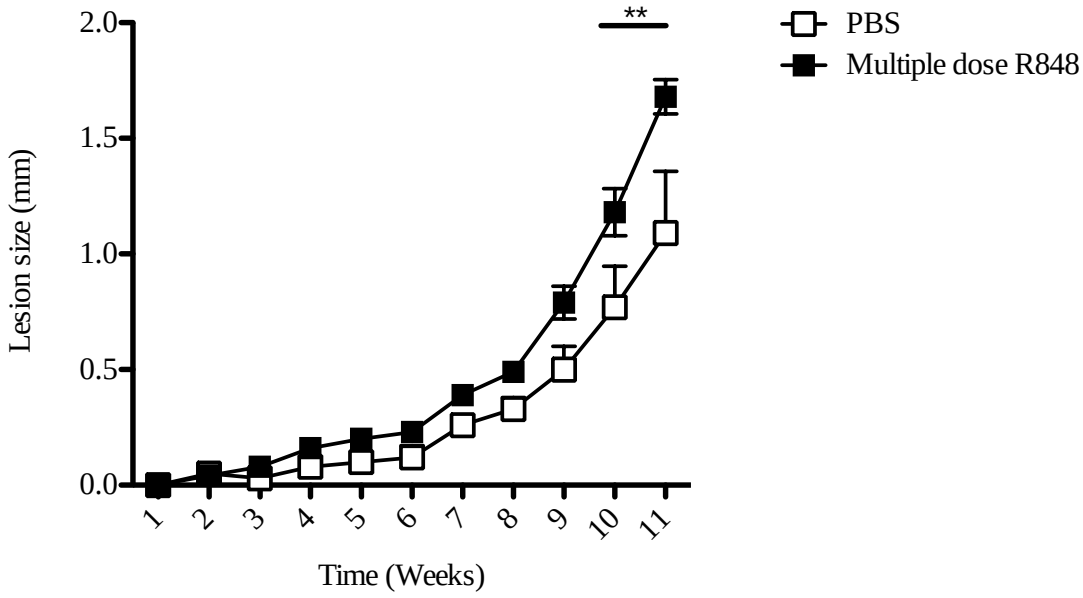


Figure 4.7. Repeated treatment with R-848 exacerbates lesion growth during *Leishmania mexicana* infection. R-848 (25 ng) was administered immediately after, 1 week, 2 weeks post infection with $2.5x10^6$ *L. mexicana* metacyclic promastigotes by s.c. injection into the hind footpads of BALB/c mice. The lesions were measured as described in **Figure 4.3**. Results show the mean ± SEM of a group of 5 mice. Statistical analysis performed using 2-way ANOVA with Bonferroni post-test. **$p \leq 0.01$.

To determine whether enhanced lesion development correlated with increased parasite burden, the lesion was excised post-mortem and prepared for parasite enumeration (Figure 4.8). Limiting dilution assay results suggested that there were no significant differences in parasite burden following multiple doses of R-848 following *L. mexicana* infection. In addition, haematoxylin and eosin staining of

lesion cryosections indicated a similar level of immune cell infiltration to the site of infection, irrespective of R-848 treatment (Figure 4.8). This evidence suggests that whilst there is a significant increase in lesion size, following administration of multiple doses of R-848, there is no detectable increase in parasite burden (Figure 4.8).

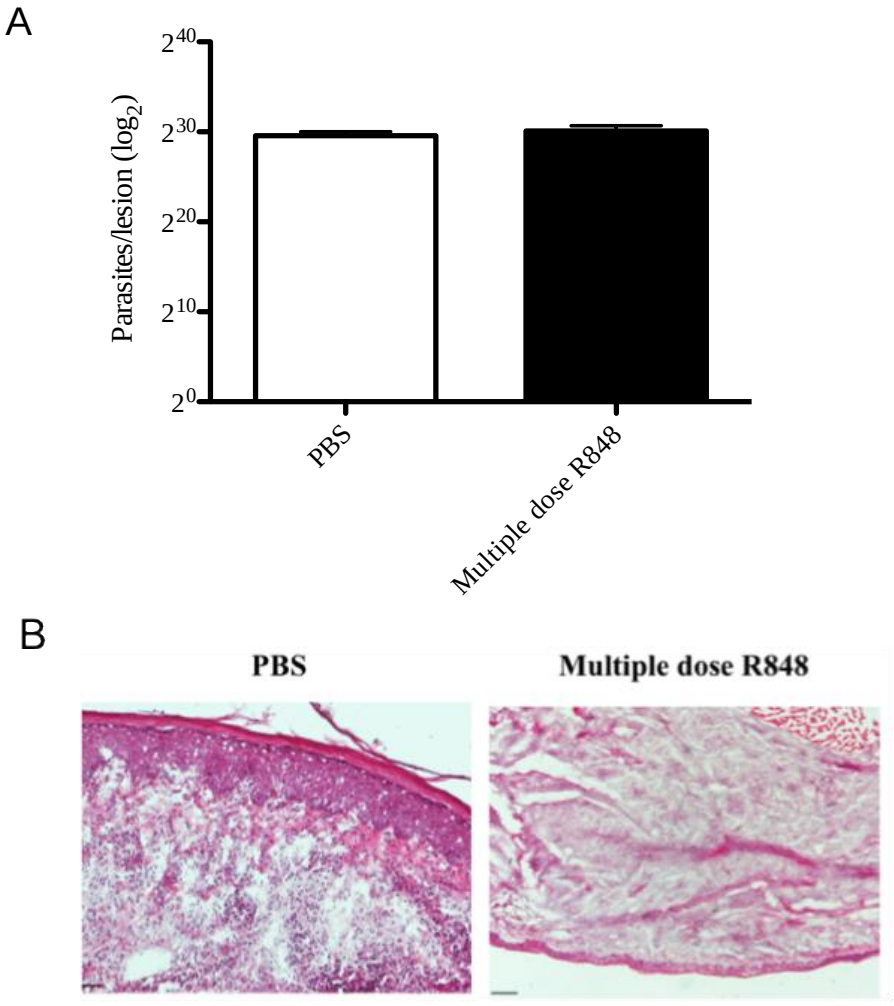


Figure 4.8. Repeated R-848 treatment does not affect lesion parasite burden during *Leishmania mexicana* infection. Mice were infected and treated as in **Figure 4.7**. The footpad lesion was either prepared for

A) parasite enumeration as described in **Figure 4.4** or processed for B) histological analysis as described in **Figure 4.5**. Results show the mean ± SEM of 3 mice. Statistical analysis performed using Mann- Whitney U test. No significant differences were identified.

The development of adaptive immunity to *Leishmania* antigens was also investigated to determine whether multiple doses of R-848 might enhance immunity. Quantification of serum antibody suggested that there were no significant differences in the level of *Leishmania*-specific IgG1 or IgG2a in mice treated with multiple doses of R-848 when compared with control mice (Figure 4.9). Further to this, splenocyte cytokine production was quantified and suggested that IFN-γ production was not significantly increased by multiple treatments with R-848, although parasite-specific cytokine production remained low overall. Together, these data suggest that multiple treatments with R-848 following *L. mexicana* infection do not further enhance the response to infection. As with a single dose, lesion development is increased by multiple injections of R-848 into the infection site, but unlike a single treatment of R-848, multiple treatments causes no significant impact to the development of adaptive immunity.

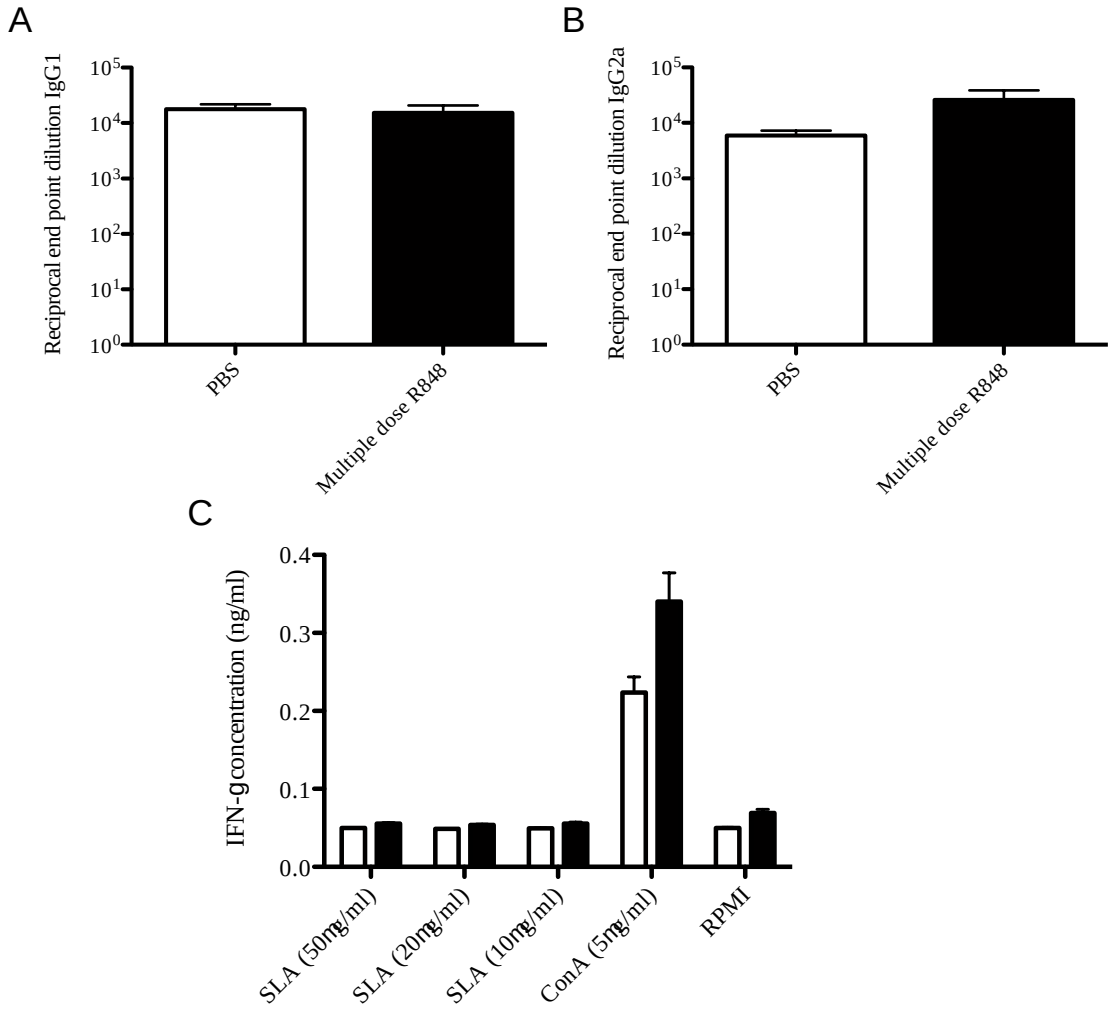


Figure 4.9. R-848 treatment does not affect IgG1 and IgG2a antibody titres or Th1-associated cytokine production. Mice were infected and treated as in Figure 4.8. A) IgG1 and B) IgG2a serum levels were determined as described in **Figure 4.6**. Spleens were prepared for splenocyte stimulation and subsequent IFN-γ production was quantified (C) as described in **Figure 4.6**. In A, B and C results show the mean ± SEM of 5 mice. Statistical analysis performed using an Unpaired T-test (A and B) and 2-way ANOVA (C). No significant differences were identified.

4.3. Discussion

Over recent years advancements in the understanding of how drugs and adjuvants can be used to specifically manipulate immune cell activation and migration have aided in the development of more efficacious treatments for a variety of infectious diseases (Bermann-Leitner and Leitner *et al,* 2014; Kumar and Engwerda e*t al,* 2014; Sathish *et al,* 2013; Petrovsky *et al,* 2004). A number of studies have demonstrated that immunomodulatory compounds can successfully direct the outcome of the adaptive immune response (Nicholls *et al,* 2010). Specifically, the toll-like receptor (TLR) 7/8 agonist, R-848, has been shown to stimulate the activation and migration of DCs *in vitro* (Ahonen *et al,* 1999; Burns *et al,* 2000; Suzuki *et al,* 2000).

It was shown in Chapter 3 that DC migration towards LV and accumulation in the draining LN is inhibited following exposure to *L. mexicana*, and that this may be associated in part with inhibition of CCR7 expression on the surface of migratory DCs. Therefore, it was hypothesised that R-848 might be useful in triggering activation and migration of DCs, thereby enhancing parasite antigen delivery to the LN and potentially altering the development of disease.

In support of previous work (Burns *et al,* 2000; Ahonen *et al,* 1999) the present study demonstrates that *in vitro* R-848 treatment will cause the upregulation of co-stimulatory factor CD40, required for antigen presentation and CCR7, essential for migration of bmDCs towards the CCL19 expressing LN, on the surface of bmDCs (Figure 4.1). Further to this, Suzuki *et al* (2000) and Tripp *et al* (2008) have demonstrated that following imiquimod application Langerhans cells migrate away from the treated skin and into the draining LN. As a result, imiquimod treated animals have an increased population of migratory LCs in the draining LN. In

addition, Yrlid *et al* (2006) suggest that feeding R-848 to rats and mice increase intestinal DC migration from the targeted intestinal tissue to the mesenteric lymph node. Indeed, the present study supports this evidence, as subcutaneous injection of 25 ng R-848 into the footpad was shown to trigger DC migration *in vivo*, leading to an increase in the number of CD11c-expressing cells detected in the draining popliteal LN (Figure 4.2). However, it is important to consider that this experiment is preliminary and does not directly measure the migration of a known population of migratory DCs, i.e. the increase in DC population found in the draining LN cannot be directly correlated with enhanced migratory capacity from the treated tissue, but may reflect enhanced DC recruitment into the LN and/or altered retention of DCs.

Recent studies have utilised immunomodulatory compounds, such as the imidazoquinolines, in a bid to enhance vaccine efficacy (Dockrell and Kinghorn, 2001; Bermann-Leitner and Leitner *et al,* 2014). Multiple *in vivo* studies have investigated the effectiveness of using R-848 as an immunomodulator primarily during viral infection (Arany *et al,* 1999; Huang *et al,* 2009), and have shown that R-848 can effectively stimulate the production of Th1 associated cytokines and IgG2a antibody generation (Reiter *et al,* 1994; Wagner *et al,* 1999; Vasilikos *et al*, 2000). As has been discussed, the development of chronic infection following *Leishmania* infection is largely associated with the lack of a sufficient Th1 type response and thus, R-848 has been identified as a potential immunomodulator for use during *Leishmania* infection (Zhang and Matlashewski, 2010; Peine *et al,* 2013; Craft *et al,* 2014).

Zhang and Matlashewski (2010), have trialled R-848 (Resiquimod) as an adjuvant for immunisation against *Leishmania major* in BALB/c mice. The authors provide

evidence to suggest that co-administration of R-848 with autoclaved *L. major* significantly increases IFN-γ production and supresses IL-4 and IL-10 cytokine production following antigenic challenge, suggesting that R-848 can skew the development of adaptive immunity towards a Th1 type phenotype and provide protection from infection.

With this in mind, the aim of the present study was to investigate how therapeutic administration of R-848 following *Leishmania mexicana* infection may influence the adaptive immune response. Other workers have used R-848 as a therapeutic during *Mycobacterium tuberculosis* BCG infection (Moisan *et al*, 2001) and demonstrated that R-848 stimulates the production of IL-12, IFN-γ and the proliferation of B cells *in vivo*.

Interestingly, Miranda-Verastegui *et al* (2005) have studied the effect of topical imiquimod treatment in combination with antimonial treatment of subcutaneous lesions of a cohort of patients with subcutaneous leishmaniasis (un-specified strains) in the clinic. The authors demonstrate that R-848 improves the cure rate for patients, when compared with those only treated with pentavalent antimony, although their well-controlled study did not reach significance. In other clinical trials of imiquimod (Aldara^{TM}), it has been demonstrated that when the drug is co-administered with meglumine antimoniate the curative rate of drug resistant cases of cutaneous leishmaniasis was increased to 90% (Arevalo *et al*, 2001). Similarly, combination treatment with imiquimod, again with meglumine antimoniate, has been shown to reduce granuloma size and is associated with modulated DC numbers found in skin (Shamsi-Meymandi *et al*, 2011). However, it was also demonstrated

that topical administration of 5% imiquimod alone does not have a significant impact on the host response (Shamsi-Meymandi *et al*, 2011).

Whilst, these results are encouraging, little is understood about how the immunological response to the parasite is affected during R-848 treatment. A number of studies have demonstrated that imiquimod can effectively stimulate macrophages *in vivo* to kill *L. major* promastigotes and enhanced nitric oxide production (Buates & Matlashewski, 1999). Additionally, it has been suggested that imiquimod (topically administered Aldara) is more effective at driving DC migration in comparision with intradermal administration (Fehres *et al,* 2014)

The results presented here support published evidence to suggest that single dosage with R-848 enhances the development of a Th1 type response. A trend was established to suggest that treatment with R-848 results in a significant increase in the generation of IgG2a antibodies (Figure 4.6) although Th1 associate cytokine production was not elevated (data not shown). These results support the findings of Zhang and Matlashewski (2010) and Moisan *et al* (2001) and suggest that single dose R-848 treatment may help towards the polarisation of the adaptive immune response, towards a healing Th1 associated phenotype.

However, surprisingly a trend was established to suggest that R-848 treatment of *Leishmania*-infected animals caused a significant increase in the subsequent development of the lesion size (Figure 4.3), and although this did not necessarily correlate with a change in lesion parasite burden, the inconsistency of parasite enumeration studies across replicate experiments (Figure 4.4) suggests that R-848 treatment does not reliably constrain parasite replication. Histological staining of the

footpad lesion (Figure 4.5) suggested that, like PBS injection, R-848 treatment resulted in an accumulation of immune cells during infection.

Initially, R-848 was administered 18h after infection. This time point was chosen to coincide with the estimated maximum migration of DCs towards the LN, during the initial period of infection, as evidenced by imaging studies presented in Chapter 3 and in a number of other studies i.e. Tripp *et al*, (2008) and Tal *et al* (2011). However, the kinetics of DC migration during the initial few days and weeks of *L. mexicana* infection have yet to be fully defined. Interestingly, it has been demonstrated that DCs interact with T cells at the lesion site as late as 2 weeks post-infection (Filipe-Santos *et al*, 2009). Therefore, it was proposed that it may be more effective for R-848 to be administered immediately after, 1 week and 2 weeks post infection. It was hoped that multiple dosage over this time period, would stimulate the maximum level of DC chemotaxis to the draining LN. The preliminary results presented here suggest that, like single dose administration of R-848, multiple administration post-*L. mexicana* infection enhances lesion development, and fails to reduce parasite burden (Figure 4.7; Figure 4.8). In addition, unlike single dose R-848 treatment, multiple dosages failed to enhance IgG2a levels or Th1 associated cytokine production (Figure 4.9). Therefore, this preliminary study suggests that multiple dosage of R-848 may not be effective in stimulating the development of protective adaptive immunity.

The study by Fehres *et al* (2014), mentioned above, would suggest that topical administration of R-848 may be more effective at initiating DC migration and T cell priming than intradermal injection. Thus, further investigation into the optimal site of administration is necessary for future work. Nevertheless, these data suggest that

early administration of R-848 may affect the development of the immune response, although, it remains unclear as to what mechanisms are at play here. In both experiments R-848 treatment resulted in enhanced lesion swelling, which was not coupled with increased parasite burden. These results suggest that immune cell infiltration to (or away) from the site of infection is somehow altered following R-848 treatment. Therefore, it is important to consider what effect R-848 injection into *L. mexicana* infected skin will have on DC maturation and migration. There is some evidence to suggest that R-848 can successfully provide environmental cues for the maturation of DCs following transfer *in vivo*. It has been demonstrated that immature moDCs injected into the skin of mice pre-treated with imiquimod, will upregulate CD11c and MHC Class II expression and migrate to the draining lymph node more effectively than *ex vivo* stimulated DCs (Nair *et al*, 2003).

In conjunction, it is possible that other immune cells present at the site of infection may be affected by R-848 treatment and subsequently influence disease outcome. As previously mentioned *in vitro* and *in vivo* studies suggest that macrophage activation and maturation is influenced by R-848. Wagner *et al* (1999) have demonstrated that macrophage derived IFN-γ and IL-12 are increased following treatment with R-848 and imiquimod (Wagner *et al,* 1999), and that subsequent T cell activation is modulated. In addition, macrophage expression levels of inflammatory chemokines, CCL1, CCL2 and CCL4 are elevated following R-848 stimulation. Thus, it is possible that R-848 may influence the local environment *in vivo* and enhance recruitment of immune cells, such as neutrophils and DCs (Sabbatucci *et al,* 2011).

In addition, it has been demonstrated that R-848 can potentiate Leishmania intracellular killing. It has been shown that treatment with the compound can

stimulate macrophage to produce higher levels of inducible nitric oxide and thus can enhance *in vitro* killing of *L. major* promastigotes (Buates & Matlashewski, 1999). This study also demonstrated that topical administration of imiquimod cream resulted in reduced lesion development and a reduced parasite burden *in vivo*.

Taken together, these studies suggest that macrophage cytokine and chemokine expression is significantly impacted upon R-848 stimulation and that this affects parasite survival and the development in disease, specifically in *L. major* infection. However, how these effects may impact the recruitment and activation of other immune cells during *L. mexicana* remains unclear.

Neutrophils will rapidly infiltrate the site of *L. mexicana* infection, and it has been demonstrated that these cells have a key role in orchestrating the recruitment of immune cells following *Leishmania* infection (discussed in Chapter 6). Interestingly, Hatterman *et al* (2007), have shown that R-848 activates human neutrophils, stimulates IL-8 production and triggers the production of PGE_2. To date, no studies have investigated how neutrophil recruitment and chemotaxis is modulated by R-848 *in vivo*, although, it is known that the mouse homologue of CXCL8, CXCL1, triggers neutrophil recruitment during *L. major* infection (Badolato *et al,* 1996) and PGE_2 is a potent inflammatory mediator (Kalinski *et al,* 2012). Indeed an increased accumulation of neutrophils into the site of a *L. mexicana* infection may increase lesion size without altering parasite growth as a recent study suggests that this parasite can enter and survive within neutrophils without inducing inflammatory mediator production (Hurrell *et al,* 2015). Interestingly, neutrophils infected with *L. mexicana* unlike those infected with *L. major* do not produce CCL3 and do not enhance DC migration into lesions.

The present study suggests that R-848 has the capacity to modulate immune cell migration, however it remains unclear as to whether DC migration is specifically targeted and if this is the driving force behind the change in antibody production following infection. To better understand the effects on R-848 treatment it would be advantageous to image the site of infection and the draining LN, in a bid to visualise DC migration following treatment. As discussed in the previous chapter, alterations in cell migration and function early infection will have a great influence on polarisation of the adaptive response, thus it would be advantageous to monitor parasite/parasite antigen containing DC migration from the site of infection to the dLN. Ideally, this dynamic process could be visualised using multiphoton microscopy or mesoscopy. Such an approach could also reveal whether treatment with R-848 (either injected or topical-application) is able to enhance this migration and overcome the failure associated with *L. mexicana* infection (Petritus *et al,* 2012)

It is also important to consider that modulation of DC migration during *L. mexicana* infection may not be limited to the inhibition of DC CCR7 expression. In fact, it is understood that there are a number of virulence mechanisms through which *Leishmania* can manipulate the host immune defenses (reviewed by Handman and Bullen, 2002; Bogdan and Rollinghof, 1998; Kaye *et al,* 2011). As has been previously discussed in Chapter 3, cysteine proteases, such as CPB, are the most likely mechanism, through which the parasite may alter DC function directly (Cameron *et al,* 2004*;* Shweash *et al,* 2011; Contreras *et al,* 2014). However, these effects may not be limited to the infected cell, as studies have demonstrated that the protease is present in the extracellular milieu (Mottram *et al,* 2004). In this way, CPB may be capable of affecting both the intracellular function of DCs and the

extracellular environment. Therefore, it may be that the failure in DC migration is associated with both cell-intrinsic and cell-extrinsic parameters. As such, whilst R-848 treatment shows a modest enhancement of parasite-specific immunity, potentially by overcoming the failure to express CCR7 on parasite-exposed DCs, it has only limited ability to overcome other immune-evasion mechanisms used by *Leishmania*. Therefore, the following Chapter sought to further explore this hypothesis.

Chapter 5. *Leishmania mexicana* derived cysteine protease B modulates DC migration by impairing CCL19 function

5.1. Introduction

Protozoan parasites have evolved over millions of years in tandem with the human immune system; as such their ability has been honed to evade the host adaptive immune response. *Leishmania spp*. employ a range of virulence mechanisms to manipulate and evade the host response. Various adaptations such as gp63, LPG and LACK expression as well as A2 gene cluster presence have contributed to the success of the pathogen (Handman and Bullen, 2002; Gomez-Areazeas *et al*, 2011; Zhang and Matlashewski, 1997).

Most notably, it has been demonstrated that *L. mexicana*-derived cysteine proteases (CPs) can influence the host immune response (Alexander *et al,* 1998; Buxbaum *et al,* 2003). The CPs are Cathepsin L-like proteases abundantly expressed by the amastigote forms of the parasite, to a lesser extent by metacyclic promastigotes and not expressed by procyclic promastigotes. As such, it has been suggested that these proteases are important for parasite survival in the vertebrate host (Mottram *et al,* 1996). In support of this hypothesis, De Souza Leao *et al,* (1995) show that following infection of macrophages with *L. amazonensis*, MHC Class II molecules can be endocytosed and cleaved by cysteine proteases released into the parasitophorous vacuole. Therefore, it is reasonable to suppose that CP degradation of MHC Class II could have a deleterious effect on macrophage antigen presentation and thus a profound effect on the development of T cell immunity and the subsequent course of infection.

Comprehensive research has suggested that deletion of genes encoding these proteases (*cpa* or *cpb*) in *L. mexicana* parasites can alter the development of infection

and influence protective immunity (Alexander *et al,* 1998). CPB-deficient mutant *L. mexicana* parasites (*Δcpb*) have been shown to be less infective *in vivo* and infection of BALB/c mice with these parasites results in reduced lesion growth and enhanced Th1 immunity. Further to this, infection of BALB/c mice with parasites deficient in both CPA and CPB (*Δcpa/cpb*) resulted in complete inhibition of lesion growth. Importantly, following inoculation with either *Δcpa/cpb* or *Δcpb* parasites, the expression of Th2 type cytokines is inhibited and, there is an increase in IFN-γ production (Alexander *et al*, 1998). Thus, these data suggest that CPB stimulates metacyclic promastigote and amastigote infectivity, enhances lesion growth and parasite replication and modulates the development of T cell immunity.

Cameron *et al* (2005) have demonstrated that CPB can actively inhibit the expression of a Th1 inducing cytokine. The authors show that IL-12 production is modulated by amastigote-derived CPB, primarily through inhibition of NFκB signalling in macrophages. Further to this, Pollock *et al* (2003) have suggested that the enzyme CPB2.8, shown to be a virulent component of the CPB complex, can actively stimulate Th2 immunity and exacerbate disease progression. This work suggests that treatment with purified CPB2.8 will cause enhanced IL-4 production and vaccination with the protein can cause increased lesion growth. Together, these studies demonstrate that CPB does not only impair Th1 type immunity *in vivo* (Buxbaum *et al,* 2003), but can also enhance Th2 associated IL-4 production, and in this way can exacerbate disease.

Thus, there are a number of mechanisms by which CPB may alter the induction of protective immunity, some of which may be associated with perturbed activation of

T cells. The results presented in Chapter 3 demonstrated that wild-type *L. mexicana* is able to abrogate DC migration *in vitro* and *in vivo*. Therefore, in this chapter the role of the virulence factor cysteine protease B, if any, in influencing DC migration during *L. mexicana* infection was investigated. Using a combination of CPB-deficient parasites and purified, recombinant CPB2.8, it is shown that this important virulence factor may play a role in regulating CCL19 activity and therefore in modulating DC chemotaxis during the initial stages of infection.

5.2. Results

5.2.1. BmDC activation and modulation of CCR7 expression is not regulated by CPB.

As discussed above, CPB has been implicated in the modulation of intracellular signaling and surface receptor expression in macrophages and DCs (reviewed Mottram *et al*, 2004). Therefore, it was postulated that CPB could be instrumental in the modulation of DC surface receptor expression during *L. mexicana* infection. Having previously shown that pre-exposure to wild-type (WT) *L. mexicana* causes a failure of CCR7 expression upregulation on the surface of bmDCs (Chapter 3), CPB-deficient *L. mexicana* parasites (Δ*cpb*) were used to investigate the function of these proteases in this context.

Flow cytometric analysis showed that bmDCs, pre-exposed to Δ*cpb L. mexicana* promastigotes did not stimulate a significant increase in CCR7 surface expression. (Figure 5.1). Following exposure to Δ*cpb L. mexicana* the population of CCR7-expressing cells was not significantly higher than unstimulated DCs. As expected, LPS stimulation of bmDCs resulted in a significant increase in the proportion of CCR7-expressing cells. Furthermore, additional LPS stimulation post Δ*cpb L. mexicana* infection did not enhance the population of $CD11c^{+}CCR7^{+}$ cells (Figure 5.1). Thus, though it has been demonstrated that *L. mexicana* actively suppresses CCR7 surface expression, these data suggest that this is not entirely a CPB dependent process.

Similarly, previous data suggests that whilst WT *L. mexicana* does not actively inhibit CD40 expression, *L. mexicana* exposure alone cannot trigger upregulation of

the receptor. Therefore, the potential influence of CPB on the expression of co-stimulatory factor CD40 was investigated. Using flow cytometry, it was demonstrated that DCs exposed to either Δ*cpb* or WT *L. mexicana* showed similar CD40 expression levels to unstimulated DCs, and in both cases CD40 expression was upregulated upon additional LPS activation (Figure 5.1B). These data suggest that CPB does not actively suppress CD40 expression and thus evasion of DC activation must be controlled by other factors.

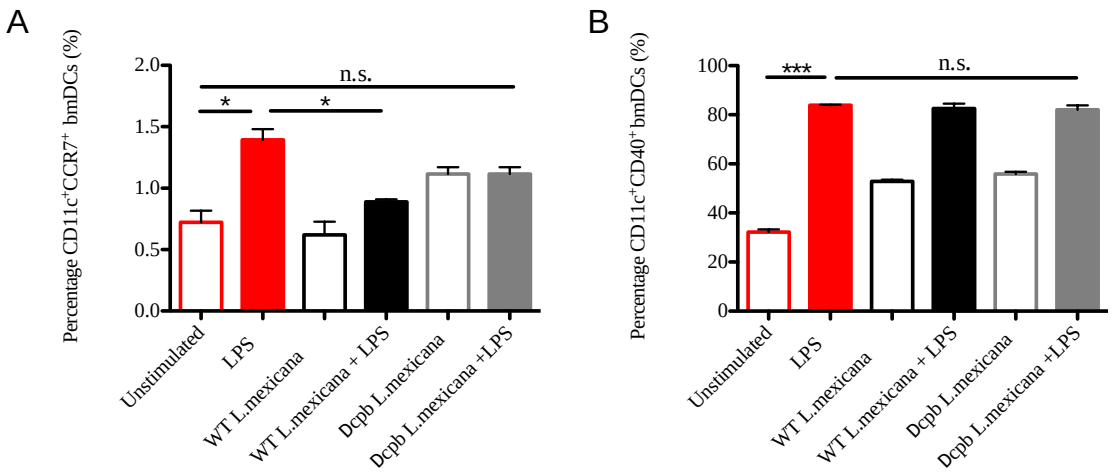


Figure 5.1. CCR7 and CD40 expression on the surface of BALB/c bmDCs is not regulated by CPB during *Leishmania mexicana* co-culture. Flow cytometry analysis of A) CCR7 and B) CD40 expression by bmDCs after 24 h of co-culture with 5:1 ratio of logarithmic stage metacyclic Wild Type (WT) or *Δcpb L. mexicana* promastigotes, and/or LPS stimulation. **Results show the mean ± SEM of 3 experiments each including triplicate samples.** Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. *= $p<0.05$. Significant differences between unstimulated and LPS stimulated; LPS stimulated

and WT *L. mexicana* exposed; LPS stimulated and WT *L. mexicana* exposed + LPS DCs. No significant (n.s.) differences between unstimulated and *Δcpb L. mexicana*/ *Δcpb L. mexicana* +LPS DCs in (A). No significant differences (n.s.) between LPS and *Δcpb L. mexicana*/ *Δcpb L. mexicana* +LPS DCs in (B).

These data demonstrate that the impaired surface expression of CCR7 or CD40 by DCs is not entirely CPB-dependent. Whilst WT *L. mexicana* can effectively circumvent DC activation and impairs DC migration, partially through reduced chemokine receptor expression, CPB does not significantly influence these processes.

5.2.2. Pre-exposure to Δ*cpb Leishmania mexicana* does not inhibit bmDC migration towards CCL19

Having shown that the level of surface expression of CCR7 by bmDCs was not significantly upregulated by Δ*cpb L. mexicana*, the 3D chemotaxis assay optimised in Chapter 3 was used to image migration of bmDC along a gradient of CCL19. As shown previously, cell tracking demonstrated that exposure to WT *L. mexicana* caused a reduction in the migratory capacity of bmDCs (Figure 5.1). Whilst this failure of DC migration may be associated with reduced CCR7 surface expression, in *L. mexicana* infected DCs, other mechanisms may be important in preventing cell motility and CPB may play an important role. Strikingly, it was demonstrated that whilst *L. mexicana*-exposed bmDCs failed to migrate along the CCL19 gradient, DCs co-cultured with Δ*cpb L. mexicana* DCs migrated efficiently (Figure 5.2). Both

the displacement and velocity of tracked DCs were significantly higher in Δ*cpb L. mexicana* treated DCs than levels observed in unstimulated DCs and in DCs exposed to WT *L. mexicana.* Moreover, whilst additional LPS stimulation of WT *L. mexicana* co-cultured bmDCs did not restore migratory DC capacity, further LPS stimulation of Δ*cpb L. mexicana*-treated DCs enhanced DC migration to such a degree that displacement values were comparable to uninfected, LPS-stimulated cells.

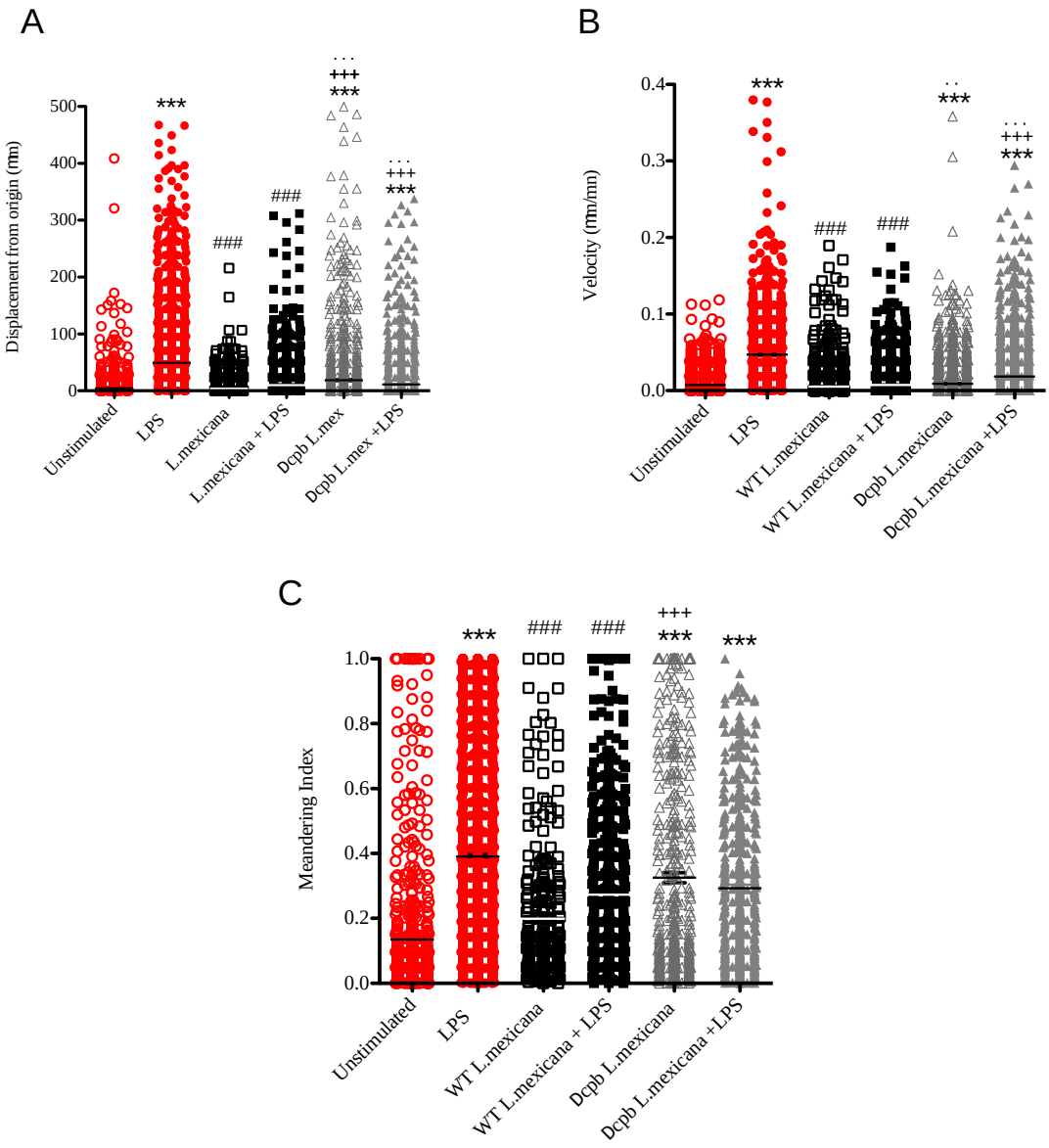


Figure 5.2. Exposure to CPB-deficient *Leishmania mexicana* parasites *in vitro* does not impair chemotaxis of bmDCs towards CCL19. BmDCs were either unstimulated, LPS (1 μg/ml) treated, or co-cultured with 5:1 ratio of logarithmic stage metacyclic Wild Type (WT) or Δ*cpb L. mexicana* promastigotes before time-lapse imaging of migration towards CCL19, as described in Chapter 3. Graphs show

quantification of A) total displacement from a point of origin, B) mean cell velocity, and C) the meandering index of individual cell tracks. These results show 3 separate experiments and show a data point for each individual cell track (100 cell sample from each independent experiment) as well as the mean (indicated by black line) ± SEM. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. Significant difference from: unstimulated DCs denoted by ***=$p<0.001$; LPS stimulated DCs denoted by ###=$p<0.001$; WT *L. mexicana* exposed DCs denoted by +++=$p<0.001$; WT *L. mexicana* exposed + LPS stimulated DCs denoted by ●●=$p<0.01$ and ●●●= $p<0.001$.

Thus, it has been demonstrated that *L. mexicana* exposure impairs bmDC migratory capacity, specifically inhibiting bmDC migration towards CCL19. Whilst, it was initially thought that the failure of DC migration was solely due to the failure of CCR7 DC surface receptor upregulation, these data using DCs exposed to Δ*cpb L. mexicana* suggest that CPB, although not associated with significant change in DC CCR7 expression, is a significant regulator of this process. DCs pre-exposed to Δ*cpb L. mexicana* parasites did not show any signs of migratory impairment, that is to say that deletion of the CPB gene resulted in complete restoration of DC migration. These data suggest that WT *L. mexicana* has a detrimental effect on the chemotactic capacity of DC during infection and CPB has a key role in this process.

5.2.3. The recombinant enzyme CPB2.8 does not directly affect DC chemotaxis, but can degrade CCL19.

The results shown above suggest that CPB-deficient *L. mexicana* does not modulate DC migration, implicating a role for CPB in abrogating cell migration. To further

investigate the role of CPB in influencing cell recruitment, a recombinant form of the CPB2.8 protein (purified from *E. coli* expression system (described by Sanderson *et al,* 2000) was used to treat cells. The specific activity (determined by the absorbance change over time during incubation of the enzyme with Z-FR-pNA, the chromogenic substrate for Cathepsins B, L, K and S, papain and trypsin) and concentration of the enzyme (determined by Bio-Rad assay) were determined and it was calculated that bmDCs would be treated with 20 μg/ml of CPB2.8 for a maximum of 1 hour, as previously described (Pollock *et al,* 2003).

Firstly, bmDCs were treated with CPB2.8 and using a combination of PI and Annexin V antibody staining, flow cytometry confirmed that CPB2.8 did not have an adverse effect on cell viability (data not shown). In previous experiments, bmDCs have been exposed to parasites and then LPS stimulated before use (Section 5.1-5.3), However, it was decided that for the next set of experiments bmDCs would first be LPS stimulated for 24 h (to induce migration) and then washed and treated with CPB2.8 for 1 h, to study the affect CPB2.8 may have on highly motile LPS activated bmDCs (as shown in Figure 5.3). The CPB2.8-containing medium was removed from the treated DCs by washing and the cells prepared for imaging using the 3D chemotaxis assay approach described previously.

Imaging and tracking of cell migration showed that treatment of LPS-stimulated DCs with recombinant CPB2.8 did not inhibit DC migration towards a CCL19 gradient (Figure 5.3) and that displacement and velocity were similar to those of bmDCs treated with LPS stimulation alone. Together, these data suggest that CPB2.8 does not directly affect the migratory function of DCs.

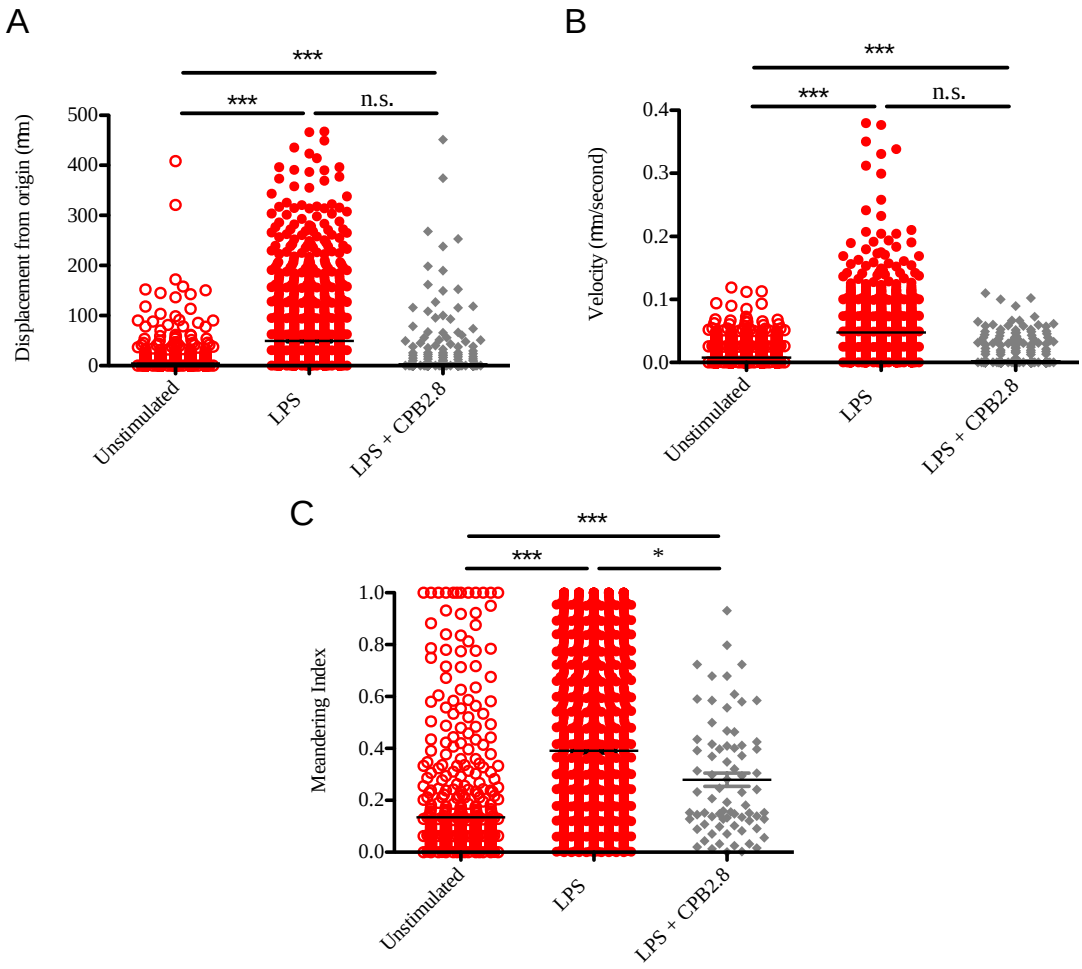


Figure 5.3. Treatment of bmDCs with purified CPB2.8 does not affect migratory capacity. BmDCs were unstimulated, LPS (1 μg/ml) treated, and treated with 20 μg/ml CPB2.8. Graphs show quantification of A) Total displacement of cell from point of origin μm) B) velocity of cellular migration (μm/minute) and C) the meandering index of the cell. These results show 3 separate experiments and show a data point for each individual cell track (100 cell sample from each independent experiment) as well as the mean (indicated by black line) ± SEM. Statistical analysis

performed using 1-way ANOVA with Bonferroni post-test. *=$p<0.05$; ***=$p<0.001$ and n.s.= no significant differences identified.

Thus, having shown that CPB2.8 does not have a direct effect on cells, it was postulated that CPB2.8 (or other parasite-derived molecules) may effectively alter the extracellular environment to abrogate DC migration, and perhaps that CPB2.8 may influence the chemokine gradient directly.

It was hypothesised that parasite population size, i.e. the amount of CPB produced, may influence DC migratory dynamics. To test this hypothesis bmDCs were co-cultured with WT *L. mexicana* at a range of different parasite to cell ratios. In the studies above (and many carried out by other workers), a 5:1 parasite to DC ratio is used during *in vitro* experiments. At this ratio approximately 96% of cells contain parasites, and therefore host cell migration may be affected directly by internalised *Leishmania* (Figure 5.4). By reducing the proportion of parasite-containing DCs, the migration of bystander, non-infected cells could also be analysed. This could potentially reveal whether the extracellular environment, such as the CCL19 gradient, is altered by intracellular parasites. Thus, DCs were co-cultured with fluorescently-labeled WT *L. mexicana* at a 1:1, 3:1 and 5:1 ratio, and prepared for imaging using the 3D chemotaxis assay. As expected, exposure to a lower concentration of parasites during co-culture resulted in a reduction in the proportion of DCs containing parasites. In a population of bmDCs exposed to 3:1 ratio, approximately 78% were parasitised, and upon exposure at a 1:1 ratio, only 55% of cell contained parasites (Figure 5.4). In this way, the migration of parasite containing and parasite-free, bystander bmDCs could be analysed.

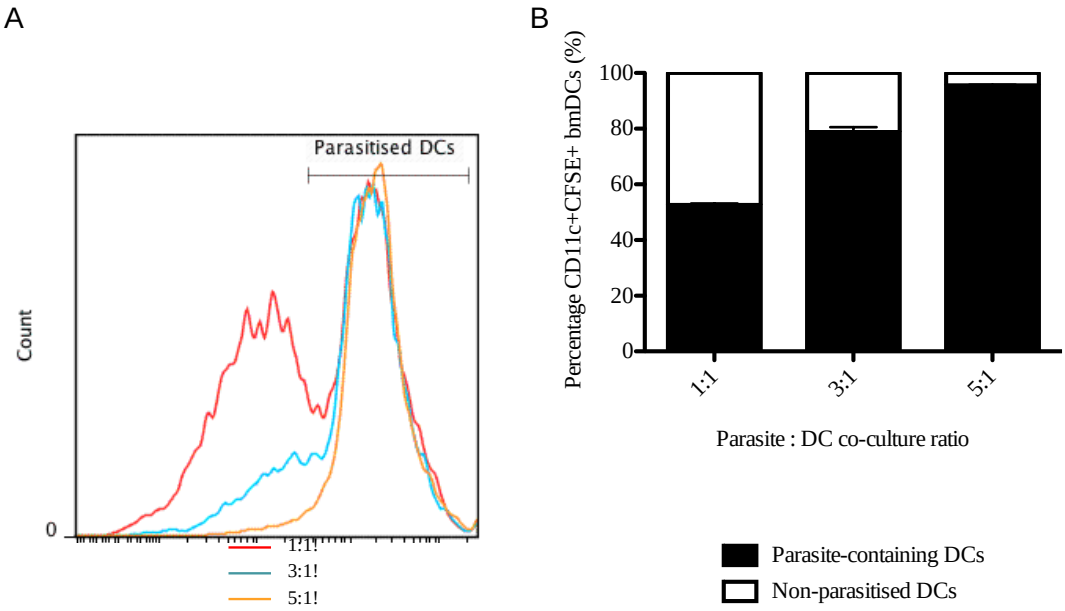


Figure 5.4. The proportion of parasitised DCs post- *Leishmania mexicana* exposure correlates with the parasite to DC ratio during co-culture. BALB/c bmDCs were exposed to 1:1, 3:1 or 5:1 CFSE labeled WT metacyclic *L. mexicana* promastigotes. 24 h after exposure DCs were prepared for flow cytometry and the proportion of $CD11c^{+}CFSE^{+}$ cells was quantified. (A) shows a representative FACs plot demonstrating the gating strategy and (B) shows the percentage of parasite-containing $CD11c^{+}$ DCs. These results are representative of 3 experiments and show the mean ± SEM.

Tracking of individual bmDCs exposed to different ratios of parasites suggested that the impairment of migratory capacity is related to the number of parasite-containing DCs. As shown previously, a DC population exposed to WT *L. mexicana* at a 5:1 ratio showed very limited migration, with low displacement values (Figure 5.5A and

B). Interestingly, after 3:1 and 1:1 exposure, DC migration was significantly enhanced in the overall population. Notably, DCs exposed to parasites at a 1:1 ratio created longer, directional cell tracks towards the CCL19 gradient (Figure 5.5A), with significantly higher displacement than DCs cultured at 5:1 ratio (Figure 5.5B). In fact, these cells had a similar displacement value to those observed in uninfected LPS-stimulated cells in Chapter 3.

By separately analysing migration of bmDCs, that either contained CFSE-labeled WT *L. mexicana* or remained uninfected, it was possible to investigate any bystander effects upon cell migration. These data demonstrated that at all ratios of parasites to DCs, individual DCs containing live parasites (green) failed to show significant migration, whereas the non-parasitised DCs show varying degrees of chemotaxis (Figure 5.5). Importantly, these results show that the migratory capacity of non-parasitised bystander DCs is proportional to the ratio of parasite exposure. This suggests that factors excreted or secreted from parasites, or parasite-containing cells may influence the extracellular environment to abrogate cell migration. Thus, it was hypothesised that parasite-derived CPB may impair the function of CCL19 chemokine, in turn reducing the migration of bystander DCs.

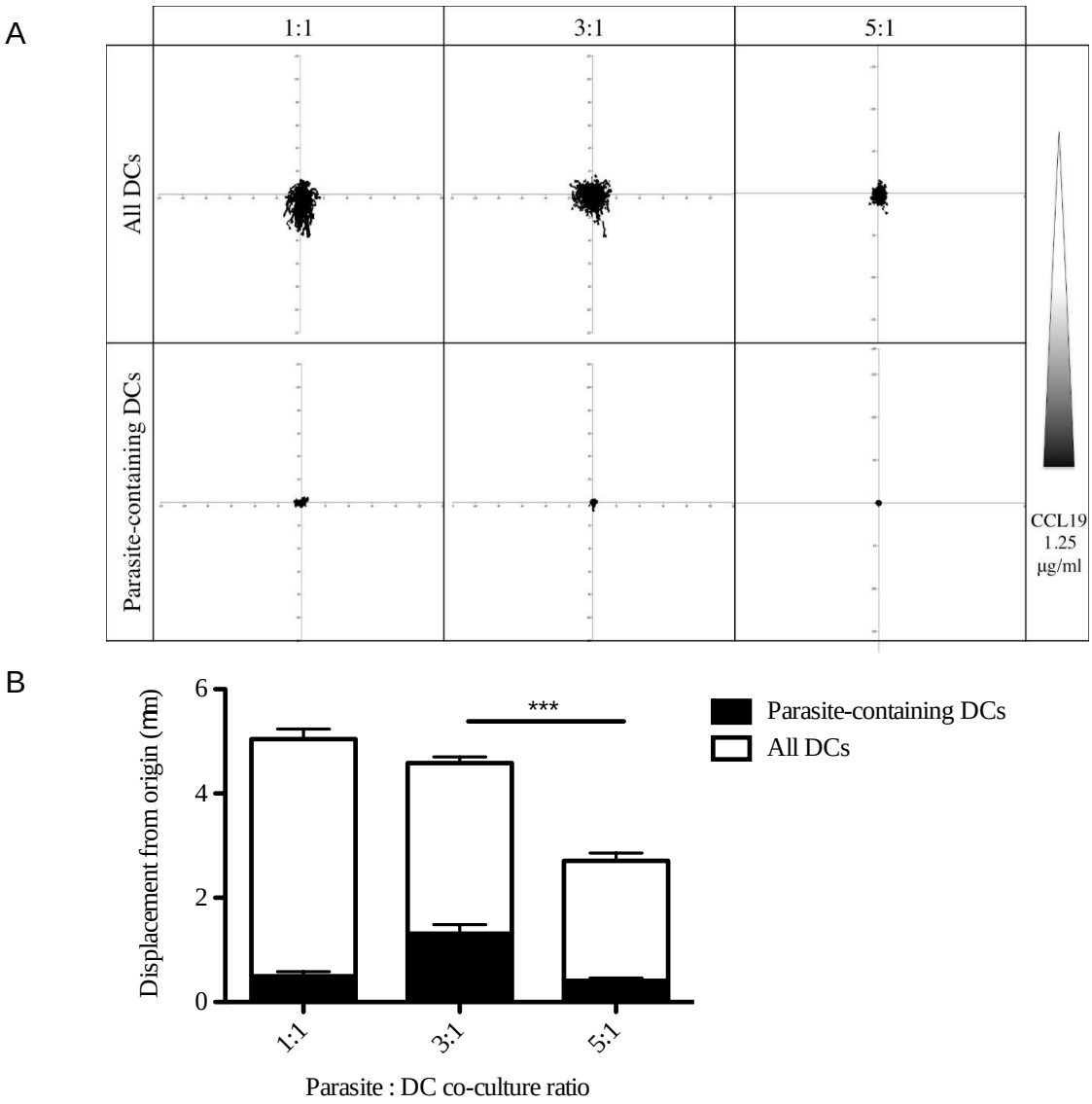


Figure 5.5. Impairment of bystander DC chemotaxis is dependent upon parasite to DC ratio during co-culture. BALB/c bmDCs were co-cultured with CFSE-labeled metacyclic wild type *L. mexicana* promastigotes at the indicated ratio of parasites to DCs. Cells were then seeded into a 3D imaging chamber and migration towards CCL19 quantified, as described above. The individual cell tracks were generated in Volocity by quantifying migration of the entire DC population (A; top row) or of $CFSE^{+}$ cells only (bottom row). B) Graph shows the

quantification of the differences in displacement between all DCs and parasitised DCs in each condition. Statistical analysis performed using 1-way ANOVA with Bonferroni post-test. Statistical differences identified between All-DC populations where ***= $p<0.001$.

To test the hypothesis that CPB might directly affect CCL19 function, the recombinant protein CCL19 used to create the chemokine gradient in the 3D chemotaxis μ-slide was pre-incubated with CPB2.8. Based on the specific activity and concentration of the enzyme, it was determined that 0.1μg recombinant CCL19 chemokine should be mixed with 1.936μg CPB2.8 and incubated at 37°C for 15 minutes, in an effort to achieve total cleavage of this amount of protein. LPS-stimulated DCs were seeded into the 3D chemotaxis μ-slide gel, and either CCL19-treated or non-treated CCL19 was used to provide a chemokine gradient. The results demonstrate that CPB2.8 treatment of CCL19 significantly reduces the chemotactic function of the molecule (Figure 5.6.). Whilst LPS-stimulated bmDCs have a high degree of displacement, forming long straight and rapid tracks towards native CCL19, the same DCs fail to migrate along a gradient of CCL19 that has been pre-treated with CPB2.8.

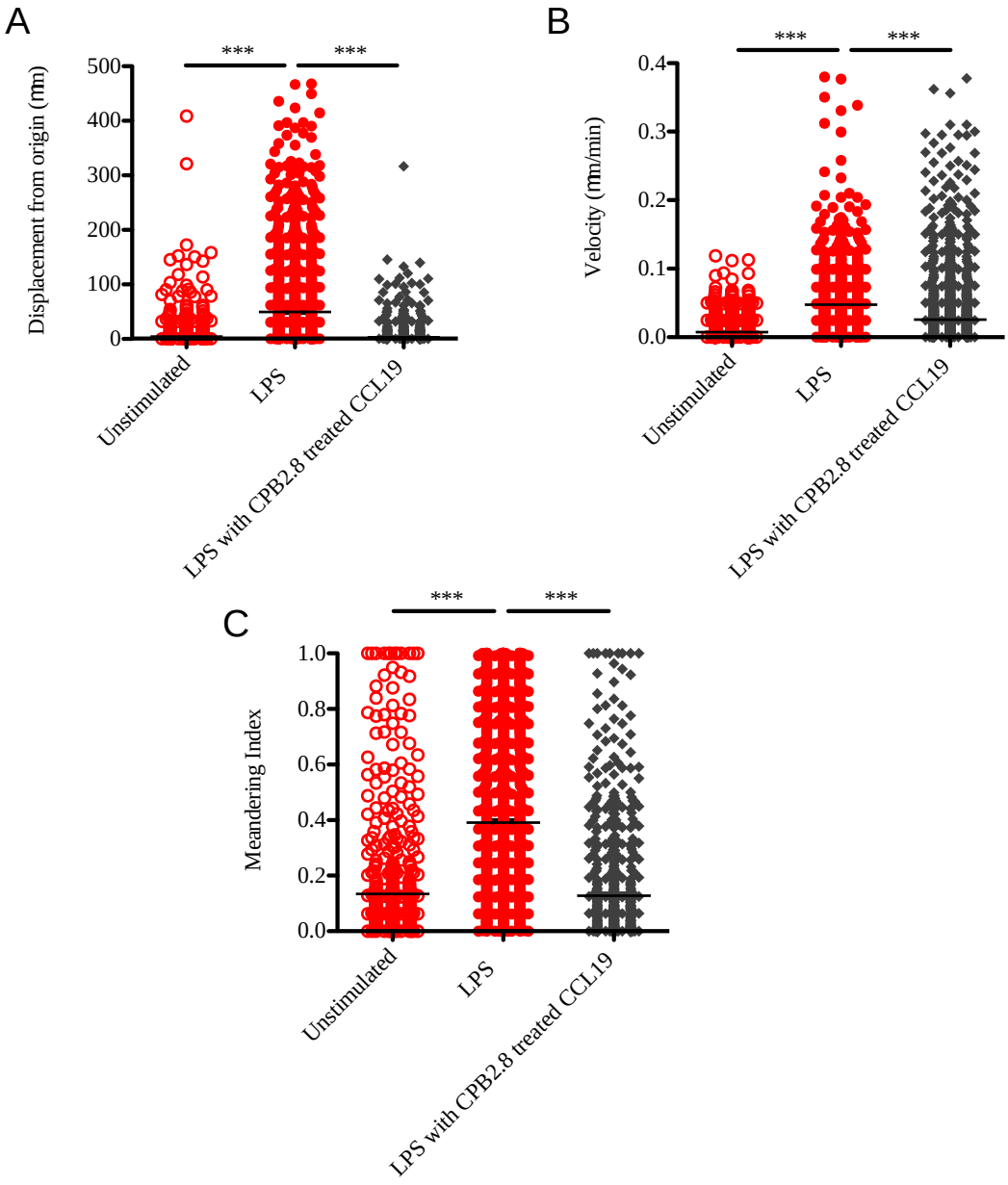


Figure 5.6. Pre-treatment of CCL19 with CPB2.8 inhibits chemoattractive function. Data generated by time-lapse microscopy of BALB/c bmDCs migrating towards a soluble gradient of CPB2.8 pre-treated CCL19 in collagen matrix gel as described previously. BmDCs were unstimulated, or LPS (1 μg/ml) treated. Graphs show quantification of A) Total displacement of cell from point of origin μm) B) velocity of cellular migration (μm/minute) and C) the meandering index of the cell.

These results show 3 separate experiments and show a data point for each individual cell track (100 cell sample from each independent experiment) as well as the mean (indicated by black line) ± SEM. Statistical analysis performed using 2-way ANOVA with Bonferroni post-test. ***= $p<0.001$.

Taken together with earlier results, these findings suggest that CPB2.8 does not directly modulate DC migration, but rather impedes the migration of the cells by disrupting the chemokine gradient. Indeed, these data demonstrate that treatment with CPB2.8 will significantly impair the function of CCL19.

5.2.4. SDS-PAGE electrophoresis suggests that CCL19 is cleaved by CPB2.8

Having shown above that CPB2.8 does not directly affect DCs migration, but rather abrogates DC chemotaxis by disrupting the CCL19 gradient, it was next important to determine if CPB2.8 could actively degrade the chemokine. While it has been demonstrated that prior incubation with CPB2.8 will render the chemokine unable to chemoattract LPS-activated DCs (Figure 5.6), it is not clear how the enzyme affects the functionality of CCL19. Therefore, it was hypothesised that parasite-derived CPB enzyme, which is Cathepsin L-like in structure (Souza *et al,* 1992; Mottram *et al.,* 2000), may degrade the CCL19.

Initially, 'SitePrediction' software (Verspurten *et al,* 2009) was used to predict the putative cleavage sites through which CPB2.8 may degrade CCL19 (mouse) peptide (UniProt), using Cathepsin L as a model protease.

Results:

>sp_O70460_CCL19_MOUSE_C_C_motif_chemokine_19_OS_Mus_musculus_GN_Ccl19_PE_1_SV_1_

rank	position	site	N fragment	C fragment	frequency score	similarity maxscore	similarity maxsite	average score	specificity
1	88 to 93	RRLK.KS	10.1 kD	1.8 kD	13.503	40.741	KAARKS	550.126	>99%
2	89 to 94	RLKK.SS	10.2 kD	1.7 kD	11.111	38.462	AARKSA	427.350	>99%
3	48 to 53	KAFR.YL	5.5 kD	6.4 kD	5.879	34.375	EAYRRF	202.087	>95%
4	22 to 27	PTLG.GA	2.7 kD	9.2 kD	5.401	36.667	KIIGGE	198.045	>95%
5	7 to 12	PLLA.FS	1.1 kD	10.8 kD	2.881	56.000	KLLAVS	161.317	>95%
6	45 to 50	NIVK.AF	5.1 kD	6.8 kD	2.083	41.379	HLVEAL	86.207	>95%

Figure 5.7 Excerpt from full SitePrediction analysis of putative cleavage sites of CCL19 (mouse) (UniProt) by Cathepsin L (Human). Scoring was carried out using the BLOSUM 62 matrix. Black boxes highlight putative cleavage positions and specificity score.

To further test this hypothesis, recombinant CCL19 was incubated with CPB2.8 (as in the previous experiment). Equivalent amounts of CCL19 and CPB-treated CCL19 were then loaded onto an SDS-PAGE gel, which was subsequently analysed by silver-staining to reveal protein bands (Figure 5.8).

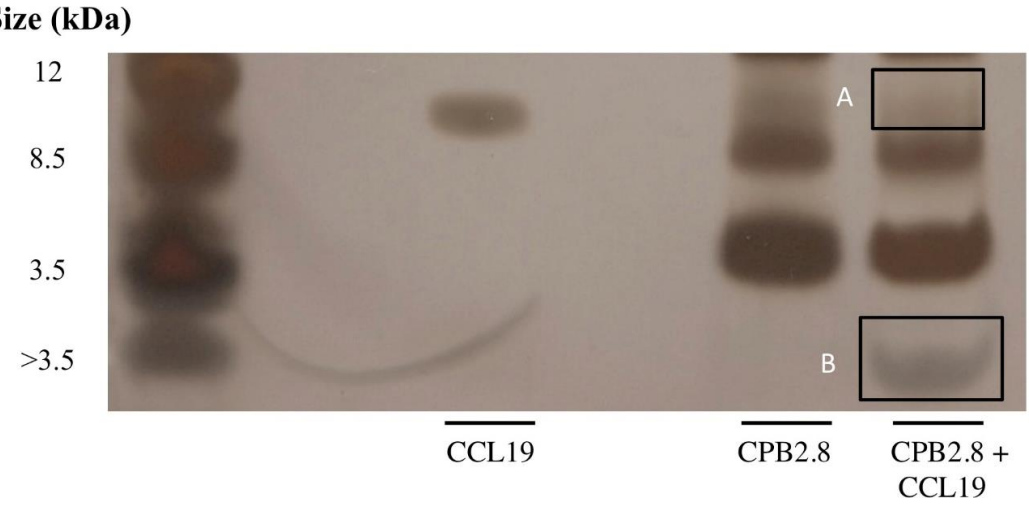


Figure 5.8. Preliminary analysis of CPB2.8-treated CCL19 by SDS-PAGE. The indicated wells of a 12.5% Glycine gel were loaded with 162.5 ng CCL19, 2.34 µg CPB2.8 or a combination of both (the same treatment ratio used in Figure 5.6 – 162.5 ng CCL19 + 2.34 µg CPB2.8)

and staining of gel was carried out using Silver Stain Kit (Pierce). Molecular mass standards (GE) are indicated.

Although not conclusive, this preliminary experiment suggested that CCL19 may be cleaved by CPB2.8. Silver staining of the SDS-PAGE gel revealed a single band of CCL19 at the expected size of approximately 9kDa. Whilst the recombinant CPB2.8 protein showed multiple bands, indicating inefficient purification, a 15 minute incubation of CCL19 with CPB2.8 caused a shift of this 9kDa band. (Figure 5.8; Box A.). In addition, protein fragments smaller than 3.5kDa were detected in this CCL19 + CPB2.8 sample (Figure 5.8; Box B). It is possible that these proteins could be fragments of cleaved CCL19. Whilst further investigation is required to validate this preliminary result, this observation indicates that *L. mexicana* CPB2.8 can directly cleave CCL19, abrogating the chemotactic capacity of this chemokine and explaining the failure in DC migration associated with *Leishmania*.

5.3. Discussion

Unlike *L. major* infections that are self-limiting, in certain mouse strains, *L mexicana* infection results in non-healing chronic infections in virtually all mouse strains. Numerous mechanisms have been suggested as to how *L. mexicana* may circumvent, evade or subvert the immune response. These include the inhibition of macrophage and dendritic cell IL-12 production (Cameron *et al,* 2004; Sweash *et al,* 2012) and the subsequent generation of a Th1 response (Kima *et al*, 1996; Wolfram *et al*., 1996; Contreras *et al,* 2014), the induction of IL-4 and a type-2 response (Pollock *et al,* 2003), the inhibition of the processes associated with macrophage and DC antigen presentation (Contreras *et al,* 2014) and interference with the cell activation processes necessary to generate an adaptive immune response (de Souza *et al,* 1995; Cameron *et al,* 2004).

A number of studies have shown that *L. mexicana* CPB, that is only expressed in metacyclic promastigotes and amastigotes, is a potent immune-modulator during infection (Alexander *et al,* 1998; Frame *et al,* 2000; Pollock *et al*., 2003; Denise *et al.,* 2003), and mice infected with CPB-deficient parasites are able to control infection and develop an enhanced type-1 response. The mechanisms by which *L. mexicana* CPs and CPB, in particular, may promote disease have been shown to be multi-factorial and include inducing IL-4 and type-2 immunity (Pollock *et al*, 2003; Denise *et al,* 2003) and degradation of macrophage NFκB to inhibit IL-12 production and a Th1 response (Cameron *et al,* 2004). In addition a body of evidence would suggest that *L. mexicana* complex CPs also degrade macrophage MHC Class II and antigen presentation (de Souza Leao *et al*, 1995; Kima *et al*, 1996; Wolfram *et al*, 1996), although they would appear to be less influential in limiting presentation

of exogenous, bystander antigens by infected dendritic cells (Bennet et al 2003). The present study would indicate that CPB may also function to interfere with the chemotaxis of DCs and thus the cell migration that is necessary to induce an adaptive immune response.

It is not uncommon for *Leishmania* virulence factors to be multi-factorial in their activity and the surface moieties lipophosphoglycan (LPG) and metalloproteinase GP63 have both been identified as having numerous activities that promote virulence not only in the mammalian but also often in the insect host (Satoskar *et al,* 1999; Handman and Bullen, 2002; Kaye *et al,* 2010). LPG has been demonstrated to enable metacyclic promastigotes to evade complement mediated lysis, to bind to TLR-2 and TLR-4 and inhibit the induction of pro-inflammatory cytokines and NO production, to inhibit phagosome-lysosome fusion and also the production of oxygen radicals (Olivier *et al,* 2005). GP63 has been associated with the degradation and resistance to antimicrobial peptides, complement mediated lysis, survival within the macrophage phagolysosome, and the interference with host cell signaling pathways (Descoteaux and Turco, 1999; Forestier *et al,* 2015).

While LPG and GP63 have both been associated with virulence in *L. major* and *L. mexicana* infections, CPs as potent virulence factors appear to be specific to the mexicana complex and infection with *L. major* modified to express *L. mexicana* CPB are more virulent than WT *L. major* and induces less IFN-γ in infected C57BL/6 mice (Buxbaum *et al;* 2003; Denise *et al,* 2003; Pollock *et al,* 2003).

Studies on LPG and GP63 that highlighted them as virulence factors were undertaken originally using purified, synthetic or recombinant molecules and host

cells *in vitro* as appropriate, and more recently in a more physiological context using gene deficient parasites that have been used generally to strengthen earlier studies (reviewed Silva-Almeida *et al,* 2012). By contrast the role of CPs as virulence factors was demonstrated initially using gene deficient or transgenic parasites, and recombinant CPs have been used to aid in determining the mode of action (Alves *et al*, 2001; Sanderson *et al,* 2001; Bauxbaum *et al;* 2003; Denise *et al,* 2003; Pollock *et al,* 2003).

To date the CPs of the mexicana complex parasites have been shown to mediate their activity at various sites to include the parasitophorous vacuole, the host cell cytoplasm and the extracellular milieu (Mottram et al 2004). De Souza Leao *et al* (1995) have show that during trafficking, MHC Class II molecules can be endocytosed and cleaved by cysteine proteases released from the parasites into the PV. CPB entering the host cell cytoplasm has been demonstrated to degrade NFκB and inhibit IL-12 production. Whilst it has been demonstrated that CPB is released into the PV by parasites, mechanisms by which CPB access the host cell cytoplasm remain to be identified. Recent studies suggest a number of mechanisms by which this could occur. For example, a cytolysin, named Leishporin, has been identified that can bind to phospholipids and is activated in the protease rich acidic environment of the parasitophorous vacuole to disrupt host cell membranes (Noronha *et al,* 2000; Almeida-Campos and Horta, 2000; Almeida-Campos 2013). This would allow diffusion of CPB not only into the cytoplasm, but also the extracellular environment. In addition, exosomes released into the PV by the parasite can fuse to the PV membrane to release contents (Silverman et al 2010) such as GP63 (Hassani et al 2011) into the host cell cytoplasm, which has also been shown to rapidly enter

the host cell cytoplasm via lipid raft microdomains (Contreras *et al*, 2010). CPB is also found in large quantities locally in lesions in the extracellular milieu (Ilg *et al* 1994), a process that could come about via the lysis of infected host cells (Mottram *et al*, 2004) perhaps facilitated by the activity of leishporin. Significantly, *in vivo* treatment of mice with recombinant CPB has been shown to specifically cleave CD23 (low affinity IgE receptor) and CD25 (IL-2R) and induces IL-4 and a Th2 response (Pollock *et al,* 2003).

The presence of large quantities of CPB in the lesion extracellular milieu in the context of the present study would suggest that an enzyme active gradient is formed from the lesion, facilitating direct degradation of CCL19. A mechanism such as this could account for the limited CCL19 mediated-recruitment of other DCs dendritic cells as described by Petritus *et al* (2012) during early *L. mexicana* infections that is implicated in the poor development of a protective adaptive immune response against this parasite. Further evidence to support that CPB in the extracellular milieu is responsible for such an effect is demonstrated in the present study by not only the significant inhibition of the migration of WT *L. mexicana* infected DCs but also of bystander non-infected DCs along a CCL19 gradient.

Sanderson *et al* (2000) and Pollock *et al* (2003) have previously purified and identified the active enzyme CPB2.8 as a key virulence factor. Subsequently, the present study demonstrates that treatment with CPB2.8 (Figure 5.7) will significantly impair the function of CCL19 and cleavage site prediction software, together with gel-electrophoresis (Figure 5.8) demonstrates that it is likely that Cathepsin L like - CPB2.8 (Souza *et al,* 1992) can effectively cleave CCL19.

However, further investigation is required to validate these results. Indeed, in order to detect low concentrations of very small proteins (<9kDa), a 12.5 % glycine gel was stained using silver stain. As this method does not allow for identification of separated proteins, a more specific method of protein detection is required. Previously, mass spectroscopic analysis of the sample was attempted, but due to the size of the protein this method of detection was unsuccessful (data not shown). In future, it is hoped that protein specific gel electrophoresis methods, such as western blotting, will be effective.

Nevertheless, if CPB can effectively degrade CCL19, it is possible that other chemokines may be similarly affected. The expression of chemokines, such as CCL2 and CCL3 (Teixeira *et al*, 2005) in the early stages of *Leishmania* infection, and during the development of the lesion (de Moura *et al,* 2005) are important for the recruitment of macrophages, DCs and T cells to the lesion site (Ritter *et al,* 2002). Thus, potential CPB mediated disruption of these chemokines may have a further effect on the instruction of an adaptive response, specifically during the latter stages of infection and therefore should be investigated in future.

It is also important to consider that while wild-type *L. mexicana* down-regulates DC CCR7 expression and fails to upregulate CD40 expression (a mechanism used by several organisms, eg. *Salmonella* and Influenza to influence DC migration and activation), not only do CPB-deficient *L. mexicana* fail to enhance DC CCR7 surface expression (Figure 5.1), but pre-treating DCs with enzymatically active recombinant CPB also fails to inhibit their migration in a CCL19 gradient (Figure 5.6). This demonstrates not only that CPB neither functions through modulating host cell signalling functions nor enzymatic cleavage to limit surface expressed of CCR7, but

that another as yet unidentified parasite factor is responsible for this down-regulation. Nevertheless the fact that DCs infected with CPB deficient parasites continue to migrate along a CCL19 gradient (Figure 5.3) indicates either that CCR7 expression remains sufficiently high to facilitate migration in DCs infected with CPB deficient parasites, that the *in vitro* assay is not sensitive enough to detect any diminished response, or that there is a level of potential redundancy in the interaction and other as yet unidentified receptors for CCL19 are present. In addition, it is interesting to note that Hansonn *et al* (2006) have shown that despite comparatively low surface expression of CCR7 following infection with *H. pylori* compared with *E. coli*, the DCs migrated equally well towards CCL19 after stimulation with either bacterium, Thus, it is possible that inhibited CCR7 surface expression alone may not be sufficient to inhibit DC migration during *L. mexicana* infection, further supporting the observations in Chapter 4.

Nevertheless, the data presented here provides evidence to support that CPB has a role in the impairment of bmDC migration towards a CCL19 gradient *in vitro* and that this is due to CPB2.8 mediated inhibition of CCL19 function. Interestingly, this mechanism of immune evasion has also been demonstrated for Poxvirus orf virus that has been shown to secrete chemokine binding protein (CBP) which binds to CCL19 and 21 and inhibits DC migration to sites of inflammation and lymph nodes (Lateef *et al*, 2010). Further to this, it is demonstrated that CPB is excreted into the extracellular milieu and that this protease will degrade CCL19 in the DC microenvironment. Thus, having disrupted the CCL19 gradient the CPB impairs DC migration of both parasitised and bystander DCs. It has previously been demonstrated that CPB exacerbates disease and modulates T cell immunity during

infection (Alexander *et al*, 1998, Pollock *et al,* 2003, Buxbaum *et al,* 2003). Thus, having described a novel role for CPB in the modulation of DC migration, it is possible that the failure to develop a robust Th1 response is in part, due to CPB-mediated impairment of DC chemotaxis and reduced DC -T cell interaction.

Chapter 6. The role of neutrophils in *Leishmania mexicana* infection

6.1. Introduction

Neutrophils, or polymorphonuclear cells (PMN) are capable of reaching areas of tissue damage rapidly and can trigger the recruitment and migration of other key immune cells (Kumar and Sharma, 2010; Wright *et al*, 2010). Therefore, the kinetics of neutrophil influx and potential cell-parasite interactions at the site of *Leishmania* infection during the first hours and days of infection is of particular interest. Neutrophils are potentially the first cells to come into contact with the parasite and, as such, these cells begin to shape the magnitude and type of immune response generated.

It is known that as early as early as 6 hours after *L. major* infection, parasites can be found inside neutrophils (Lima *et al*, 1998). However, the outcome and purpose of neutrophil mediated parasite uptake remains unclear. Laskay *et al* (2008), have hypothesised that neutrophils can act as "Trojan horses" by engulfing *L. major* parasites, upregulating surface apoptotic markers and triggering engulfment by phagocytic macrophages, thus providing a method of "silent" parasite delivery into the definitive host cell. However, Peters *et al* (2008) have used *in vivo* imaging to show that whilst neutrophils can readily phagocytose parasites, and act as the initial host cell, there is no evidence to suggest that uptake of parasitised neutrophils by macrophages occurs. This is not to say that "Trojan horse" neutrophil uptake is not possible, however the evidence provided by Peters *et al*, (2008) supports the hypothesis that neutrophils could promote promastigote survival during the initial infection period, providing a niche for parasite development and a strategy for avoidance of cell and complement mediated killing.

In support of the hypothesis that neutrophils will shield promastigotes from the host innate immune defenses, transient depletion of neutrophils during the first weeks of *L. donovani* infection has been shown to exacerbate disease and, importantly, lead to the development of a skewed Th2 type immune response (McFarlane *et al*, 2008). However, this concept has its limitations, and it is important to note that the function of neutrophil mediated immunity has been shown to be heavily dependent upon the *Leishmania* species comprising infection and the mouse strain used. Certainly, in the case of *L. major* infection, Tacchini-Cottier *et al* (2000) have shown that depletion of neutrophils in susceptible BALB/c mice (using a monoclonal antibody known as NIMP-R14) reduces disease severity and impairs Th2 bias. Conversely, neutrophil depletion in resistant C57BL/6 mice promotes the development of more persistent disease. This evidence, serves to highlight the different roles neutrophils play in cutaneous and visceral *Leishmania* infection.

As highlighted above, the neutrophil response to *Leishmania* plays an important role in regulating the response of other immune cells. Charmoy *et al* (2010) demonstrate that depletion of neutrophils in C57BL/6 mice results in a significant impairment of CCL3 production, and thus impaired dendritic cell recruitment to the site of *L. major* infection. As has been discussed in previous chapters, a reduction in DC influx to the infection site, and thus reduced contact with parasite antigen, may affect downstream DC-T cell interactions in the early stages of infection. Nevertheless, it seems that the presence of neutrophils during initial infection could be a "double-edged sword". Whilst the cell may provide a refuge for the promastigote, protecting the parasite from extracellular killing, the neutrophil is also instrumental in recruiting antigen-

presenting cells and appears to play an important role in limiting disease in some experimental leishmaniasis models as outlined.

To add further level of complexity to the host/parasite interactions, a neutrophil can undergo a process known as NETosis, whereby the cell can physically trap and kill potentially harmful microbes by forming neutrophil extracellular traps (NETs) (Brinkman, 2007). These NETs are formed by the extrusion of neutrophil nuclear material and are primarily composed of filamentous DNA. Interestingly, it has been demonstrated that *L. amazonensis* promastigotes, which are part of the "mexicana-complex" of parasites, can initiate NET formation and that trapped parasites can be killed by leishmaniacidal histone arrangements present in extruded neutrophil DNA (Guimaraes-Costa *et al*, 2009). However, to date no studies have defined a role for NETs in mediating protection from *Leishmania in vivo* and their importance in the initiation of an adaptive immune response remains unclear.

Interestingly, during the course of this work, a study using neutropenic mice provided evidence to suggest disease-promoting role for neutrophils (Hurrell *et al*, 2015) during *L. mexicana* infection. However, the results obtained within the present study would suggest at least some neutrophil properties/functions have a protective function during infection.

In summary, there is significant evidence that the neutrophil performs many important functions during *Leishmania* infection. It is suggested that PMNs can act as primary host cells, promoting further infection; they can also orchestrate antigen presenting cell influx to the infection site and may even contribute to extracellular

killing through NET formation. However, to date, the function of neutrophils in the context of chronic *L. mexicana* infection has not been fully defined.

The aim of this chapter was to investigate the role of neutrophils and NETs in the development of chronic infections with *L. mexicana*. Initially, the kinetics of neutrophil recruitment during early *L. mexicana* infection was quantified. Subsequently, the ability of these recruited neutrophils to affect the migration of DCs into draining lymphatic vessels was investigated. Finally, using novel knockout mice incapable of NET formation, the importance of these structures in the development of chronic *L. mexicana* infection was examined.

6.2. Results

6.2.1. Neutrophils are recruited following *Leishmania mexicana* infection

As discussed above, effective neutrophil recruitment is important for the development of immunity during *Leishmania major* and *donovani* infection (Tacchini-Cottier *et al,* 2000; McFarlane *et al,* 2008; Ritter *et al,* 2009) and these "rapid responder" cells may influence other immune cells. The results presented in Chapter 3 suggest that *L. mexicana* suppresses DC migration *in vitro* and *in vivo* and therefore the main aim for this chapter was to examine whether neutrophils play a role in influencing DC migration. However, the kinetics and importance of early neutrophil recruitment have not been fully characterised in the *L. mexicana* infection model. Therefore it was important to first quantify the population of neutrophils found in the ear dermis in the first 24 h post infection with *L. mexicana* promastigotes.

In this preliminary experiment, the ears of BALB/c mice were intradermally (i.d.) inoculated with $1x10^6$ parasites or carrageenan, a xantham gum derivative that has been demonstrated to cause localized inflammation and stimulate neutrophil recruitment to the site of injection (Morris *et al,* 2003). The ears were then removed either 2 or 24 hours after injection/infection and split to form dorsal and ventral sheets for explant culture. This isolation method allows for the quantification of cells present in the ear tissue at a specific time point post infection (Belkaid *et al,* 2000). After 18 hours, the recovered emigrating cells were counted and prepared for flow cytometric analysis. Cells were identified using FSC and SSC gating and the total number of Ly6G-expressing neutrophils quantified by determining the proportion of

cells (relative to isotype controls) and multiplying by the absolute number of cells recovered from the ear explant (Figure 6.1A).

Two hours after challenge with either carrageenan or *L. mexicana* promastigotes ear tissues contained a relatively small number of neutrophils, only slightly elevated above the numbers observed in resting ears (Figure 6.1B). However, 24 hours after injection with carrageenan the number of neutrophils isolated from the injected ears was considerably elevated relative to control ears. Similarly, the number of neutrophils isolated from *L. mexicana*-infected ears was increased at this later time point.

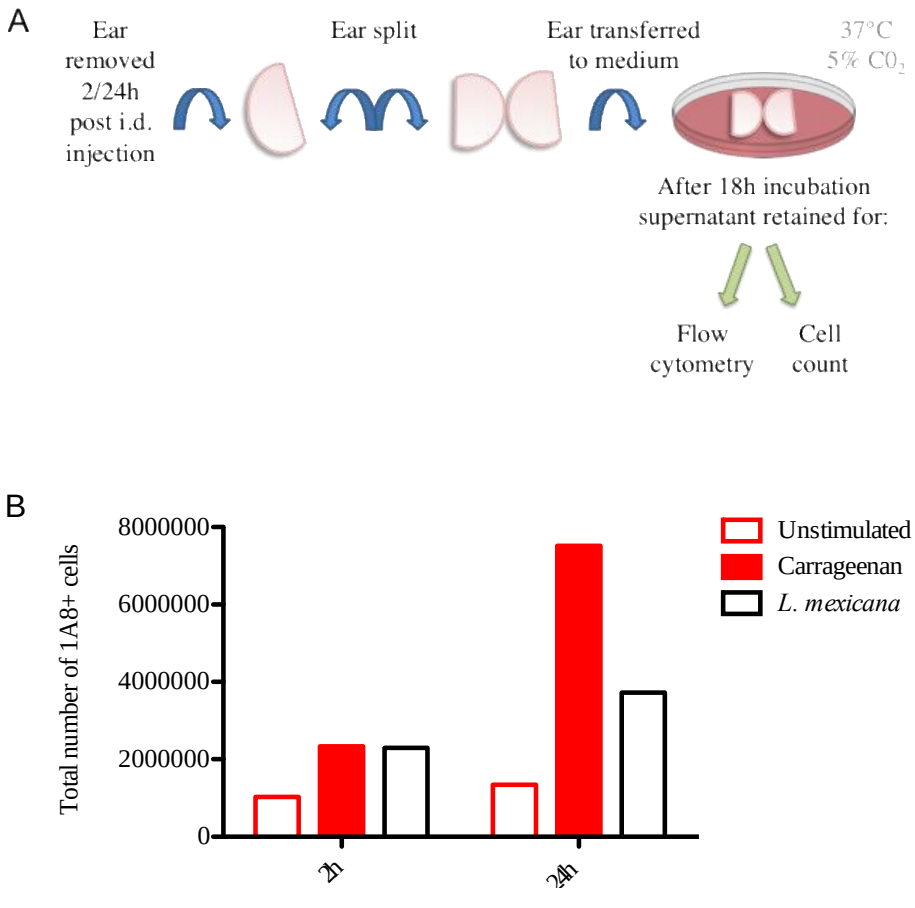


Figure 6.1. *Leishmania mexicana* infection triggers neutrophil recruitment to the infection site. BALB/c mouse ears were prepared for

explant culture as described previously (Belkaid et al, 1996). A) Schematic describing tissue preparation for culture. Ears were removed 2 h and 24 h post-intra-dermal injection of 60 μg of carrageenan or $1x10^6$ *L. mexicana* promastigotes. Naïve tissue was used a negative control sample. Explants were layered over cRPMI and cultured for 18 h. Recovered cells were counted and prepared for flow cytometric analysis. B) Total number of $1A8^+$ neutrophils in ear explants isolated 2 h or 24 h after challenge. The graph shows results from a pilot experiment of a single mouse per condition.

The clear increase in the total number of neutrophils present in the tissue at 24 h suggest that both carrageenan treatment and *L. mexicana* infection stimulates neutrophil influx into the ear dermis. Whilst the parasite does not trigger neutrophil recruitment to the same degree as carrageenan treatment, exposure to *L. mexicana* promastigotes does successfully induce neutrophil influx into infected tissue, as has been shown for other *Leishmania* species (Peters *et al,* 2008).

It must be noted that this experiment was a pilot study, intended to confirm efficient neutrophil influx into *L. mexicana* ear dermis after infection. However, whilst it was not possible to carry out statistical analysis on the obtained results the described data serves to support this hypothesis.

6.2.2. Depletion of neutrophils during early *Leishmania mexicana* infection exacerbates lesion growth.

Having demonstrated that neutrophils readily migrate to the site of infection, it was next important to qualify the influence these cells have on disease outcome.

Extensive studies have used monoclonal antibodies (such as NIMP-R14, RB6-8C5 and 1A8) (Tachini-Cottier *et al,* 2000; Ribeiro-Gomes *et al,* 2012) to transiently deplete neutrophils and have highlighted the important role of PMN in the development of *Leishmania* immunity and disease severity. Work from the Tacchini-Cottier laboratory has established that during *L. major* infection, neutrophils are important for chemokine expression and DC recruitment (Charmoy *et al,* 2008), and further work has demonstrated that during *L. donovani* infection, neutrophils have a protective role (McFarlane *et al,* 2008). However, the long-term consequences of depleting neutrophils during the initial phase of *L. mexicana* infection, and the effect this may have on disease outcome and neutrophil kinetics, has yet to be defined.

To investigate the role of neutrophils during early *L. mexicana* infection, intraperitoneal administration of 200 μg NIMP-R14 mAb was used to deplete neutrophils in BALB/c mice 6 h prior to infection in the footpad with 10^6 promastigotes. Previous reports (Lopez *et al,* 1984) have demonstrated that this method of depletion results in reduced numbers of circulating neutrophils for approximately 72 h after administration. An isotype-matched control antibody (Vac-α) was used as a negative control treatment. NIMP-R14 mediated neutrophil depletion was confirmed by flow cytometric analysis of a blood sample from a treated mouse prior to experimental usage (Figure 6.3A). The course of neutrophil influx in the infected footpad was then monitored using a non-invasive *in vivo* imaging system (IVIS), applying a luminol-based method to generate a bioluminescent signal proportional to myeloperoxidase activity, often associated with neutrophils (Gross et al, 2009). The total light emission was quantified (total flux) and used as a measure of the neutrophil population present in the region of

interest (ROI) (Figure 6.2). In parallel, disease progression, following infection was monitored by measuring increase in footpad thickness at weekly intervals (Figure 6.3). IVIS imaging of infected footpads 24 h post *L. mexicana* injection, revealed that NIMP-R14 -treated animals displayed a negligible bioluminescent signal at the site of infection (Figure 6.2B), significantly less that than their Vac-α treated counterparts, demonstrating that treatment successfully depleted neutrophils in all animals. As expected, there was a clear signal detected in the ROI of the Vac-α injected control mice showing accumulation of neutrophils at the site of inoculation and supporting the observations in Figure 6.1.

After this early influx of neutrophils, comparatively low-level signals were detected at the site of infection in both groups of mice for the first 3 weeks, suggesting that neutrophils returned to low levels. Nevertheless, it is clear that by 3 weeks post treatment, NIMP-R14-treated animals regained a functional neutrophil population and both groups demonstrated an increased accumulation of neutrophils at the site of infection. Whilst at no point in the experiment did differences in signal between the groups achieve significance, NIMP-R14 treatment appeared to slightly enhance neutrophil influx to the site of infection, when compared with the neutrophil kinetics of Vac-α treated mice. Together these data suggest that early depletion of neutrophil causes a subsequent increase in neutrophil recruitment after the cell population has recovered from NIMP-R14 mediated depletion.

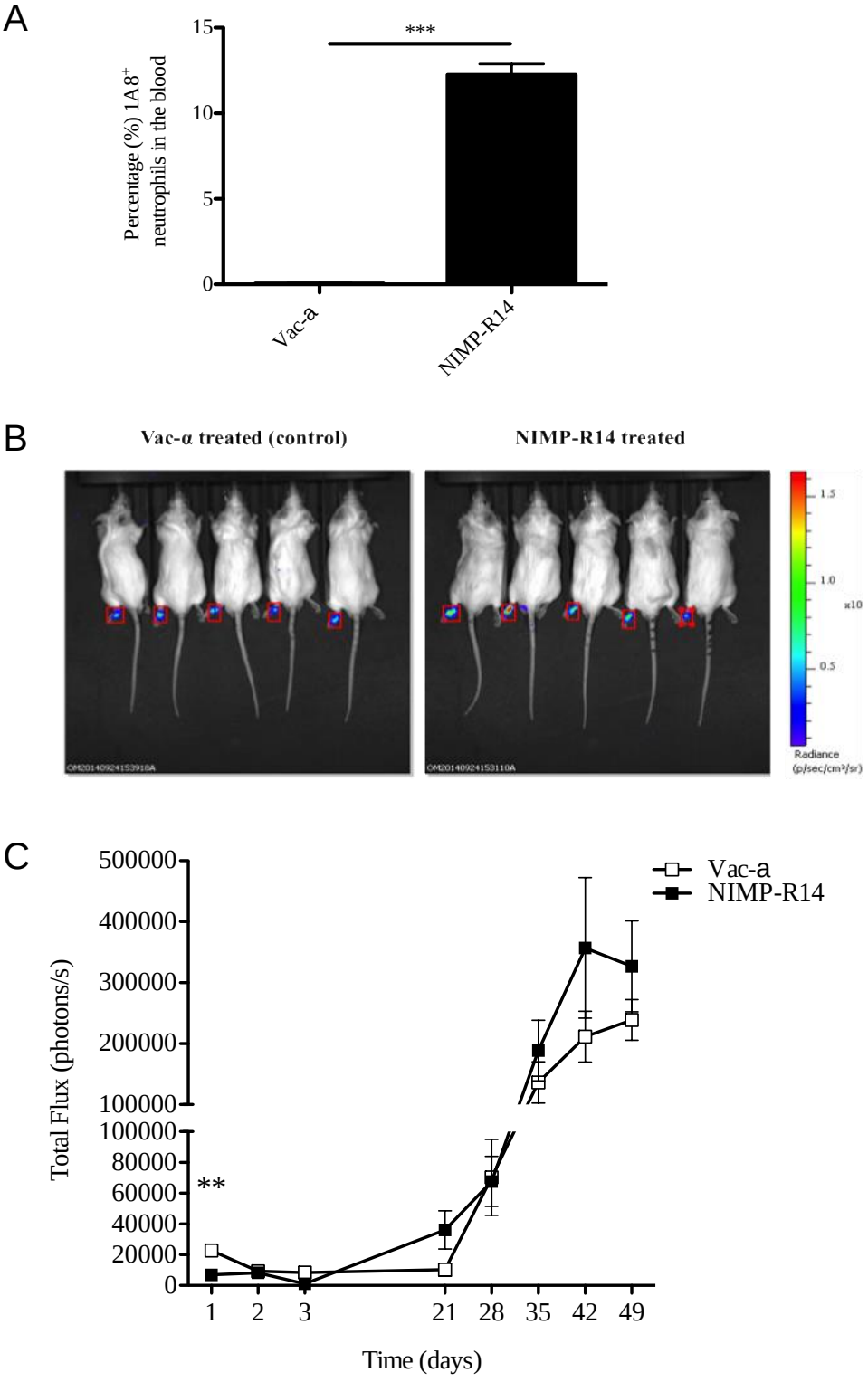


Figure 6.2. Long-term neutrophil infiltration to the site of infection is not altered by transient NIMP-R14 mAb depletion. BALB/c mice

were depleted of neutrophils with 200 μg NIMP-R14, infected into the left hind footpad with 2.5 x 10^6 *L. mexicana* promastigotes and at regular intervals, luminol (200 mg/kg) was injected i.p. to detect MPO activity, an indirect measure of neutrophil infiltration. Control mice received an isotype-matched control antibody (Vac-α). Peripheral blood samples were taken 6 h post NIMP-R14 treatment and prepared for flow cytometric analysis. A) Total number of $1A8^+$ neutrophils present in the blood. B) Representative image showing the imaging area and selected ROI (red boxes) for the Vac-α and NIMP-R14 treated groups. C) Total flux measurements (photon/s) detected within the ROI regions over a 7 week (49 day) infection period. Results represent the mean ± SEM of 5 mice per group. Statistical analysis performed using student T test. **= $p \leq 0.005$ ***=$p \leq 0.001$.

In parallel, the infected footpads of the NIMP-R14 and Vac-α-treated mice were measured over the same infection period and the lesion sizes were compared (Figure 6.3). From just 3 weeks post-infection, there was a clear divergence in lesion size between the two treatment groups. Whilst Vac-α-treated, control animals had a small degree of lesion growth over the period of infection, in line with the expected projection of disease progression, NIMP-R14-treated mice showed significantly larger lesion sizes from 6 weeks onwards.

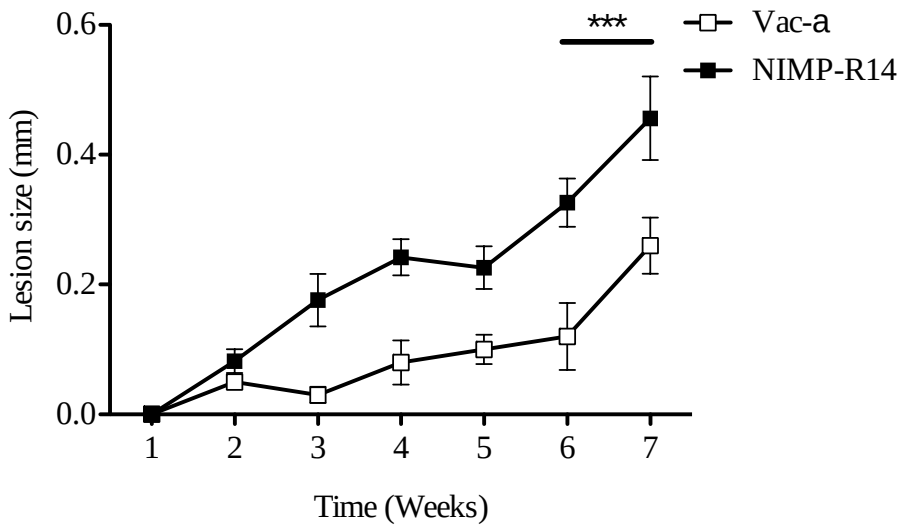


Figure 6.3. NIMP-R14-mediated depletion of neutrophils during initial *Leishmania mexicana* infection enhances lesion growth. BALB/c mice were treated with 200 μg NIMP-R14 (or Vac-α control antibody) 6 hours prior to inoculation of 2.5 x 10^6 *L. mexicana* metacyclic promastigotes into the footpad. Lesion development was measured at weekly intervals using spring-loaded calipers. Results represent the mean increase in footpad thickness ± SEM of 3 separate experiments with 5 mice per group. Statistical analysis performed using 2-way ANOVA with Bonferroni post-test. ***= $p \leq 0.001$. Statistically significant differences between Vac-α and NIMP-R14.

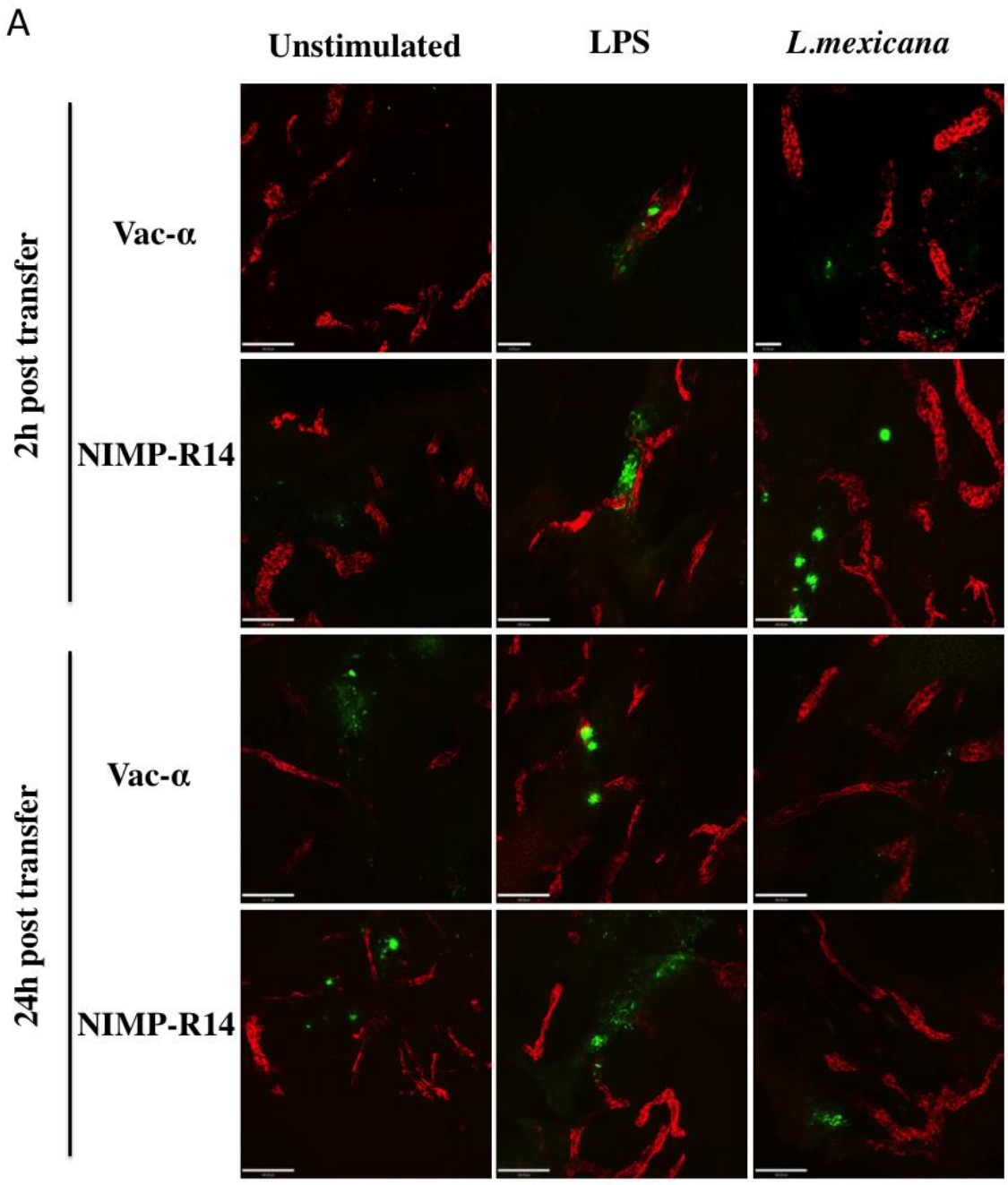
Taken together, these data suggest that a transient neutrophil depletion early in infection results in a more pronounced neutrophil influx to the site of infection at later stages and also significantly enhances lesion growth. Whilst this highlights the importance of the neutrophil in early infection, the mechanism by which NIMP-R14-treatment enhances lesion growth in BALB/c mice remains unclear. Having observed

that DC migration towards and into LV is inhibited by *L. mexicana* in Chapter 3, the objective for this Chapter was to investigate the role neutrophils may have in instructing DC migration towards the lymphatics following exposure to parasites.

6.2.3. Transient neutrophil depletion does not modulate DC interaction with the draining lymphatic vessels

The data above demonstrated that NIMP-R14 mAb mediated neutrophil depletion affects the development of disease following *L. mexicana* infection. Given that Charmoy *et al* (2010) have described that neutrophil-derived CCL3 is required for the recruitment of DCs into the infection site and that results presented in Chapter 3 suggest that DCs at the site of infection fail to migrate towards the lymph node, it was next investigated whether neutrophil depletion might affect DC migration and interaction with lymphatic vessels.

Using an approach similar to that described in Chapter 3, DC migration was imaged by confocal microscopy of neutrophil-depleted (or control) mice at various times after DC transfer. Neutrophils were depleted by intra-peritoneal administration of NIMP-R14 (or Vac-α control) 6 h prior to intradermal transfer of $1x10^5$ CFSE-labeled bmDCs that had previously been exposed to *L. mexicana* promastigotes or stimulated with LPS (as described in Chapter 3). Injected ears were removed 2 or 24 h after cell transfer, fixed and stained with eF-660-conjugated anti-LYVE-1 antibody (red) to reveal LVs. The interaction of the CFSE-labelled DCs and the LV network was then imaged using confocal microscopy (Figure 6.4A) and co-localisation quantified as in previous chapters.


A
Unstimulated
LPS
L.mexicana
2h post transfer
Vac-α
NIMP-R14
24h post transfer
Vac-α
NIMP-R14

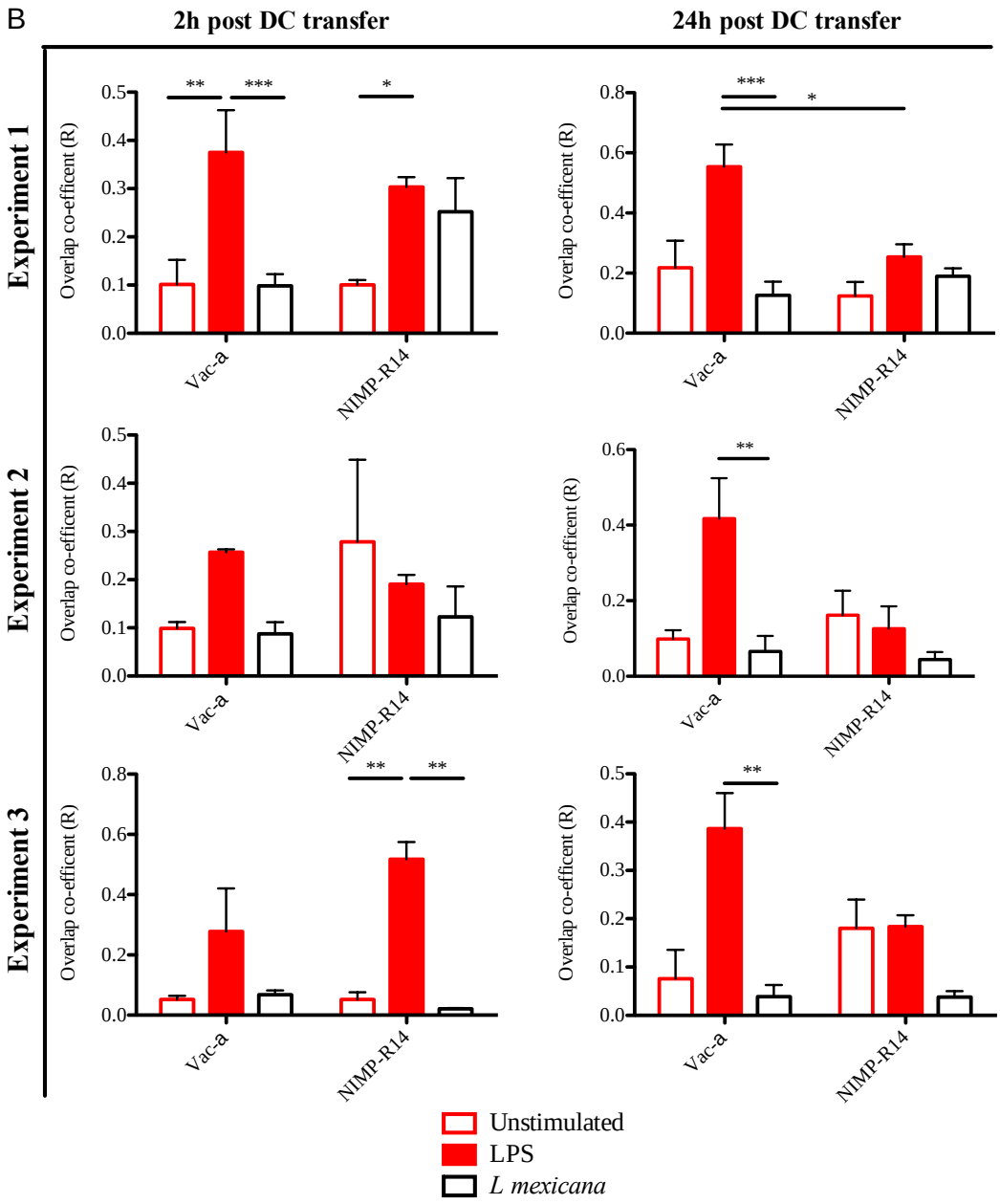


Figure 6.4. *In vivo* DC interaction with lymphatic vessels is not enhanced following neutrophil depletion. C57BL/6 mice were treated with 200 μg NIMP-R14 or Vac-α 6 hours prior to intradermal injection of 10^5 bmDCs. BmDCs were either unstimulated, LPS-treated, or exposed to *L. mexicana* promastigotes (5:1 ratio) before CFSE labeling (green) and transfer into recipient neutrophil-depleted or control mice.

Two or 24 hours later, the ear was removed, fixed, permeabilised and stained with eF-660-conjugated anti-LYVE-1 antibody to reveal lymphatic vessels (red) before imaging using confocal microscope using a x20 objective lens. (A) Three-dimensional image stacks are presented, representative of 3 mice per group are presented showing DCs (green) and LVs (red). Scale bar=160 μm. (B) Images were analysed to quantify bmDC colocalisation with lymphatic vessels (expressed as the overlap coefficient) using Volocity software. Data presented in (B) shows the mean overlap values of 3 mice per group (± SEM) for 3 separate experiments. Statistical analysis performed using a 1-way ANOVA with Bonferroni post-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Two hours after DC transfer, few unstimulated DCs had migrated towards the lymphatic vessels in either NIMP-R14- or Vac-α-treated mice (Figure 6.4A). Conversely, and as expected, LPS stimulation appears to trigger rapid DC migration towards lymphatic vessels and CFSE-labelled DCs cells appear to localise near to vessels just 2 hours after cell transfer and irrespective of neutrophil depletion. Indeed, quantification of multiple images from repeat experiments revealed a significant increase in the overlap co-efficient (a measure of DCs migrating into LVs) when comparing the LPS-stimulated DCs with unstimulated cells (Figure 6.4B).

As observed in previous experiments (Chapter 3), transfer of DCs that had previously been exposed to *L. mexicana* parasites failed to induce significant

migration towards the LV, and the few cells that are proximal show minimal translocation into the vessel. In control, Vac-α-treated mice, bmDCs co-cultured with *L. mexicana* promastigotes consistently failed to colocalise with LVs, meaning that the overlap co-efficient was similar to that of unstimulated DCs. Whilst, *L. mexicana*-exposed DCs transferred into neutrophil-depleted animals showed more variability in the extent of co-localisation between replicate experiments (Figure 6.4B: Experiments, 1, 2 and 3), the overall trend mirrored that observed in control animals and there was no significant restoration of DC migration towards LVs in the absence of neutrophils, even at later time points .

Taken together these data suggest that, in this case neutrophils do not have significant influence on DC migration towards and into LV. As discussed in Chapter 3 *L. mexicana* abrogates DC-LV contact, however, these data demonstrate that that NIMP-R14-mediated depletion of neutrophils does not reverse this effect.

6.2.4. NETs are important for parasite uptake by phagocytic immune cells during *Leishmania mexicana* infection.

Results so far in this chapter have demonstrated that the depletion of neutrophils prior to *L. mexicana* infection exacerbates disease progression. However, how and why depletion of neutrophils has such a profound affect on disease outcome remains unclear. One way in which neutrophils may influence nearby cells is through the formation of NETs, and it has been shown that *Leishmania* promastigotes can trigger NETosis, leading to efficient trapping and killing of parasites (Guimaraes-Costa *et al*, 2009). However, to date, no studies have described how NETs contribute to the

development of an adaptive immune response following *L. mexicana* infection and it is unclear whether NETosis influences how other immune cells acquire parasite-derived material. Therefore, the next objective for this chapter was to use mice deficient in NET formation to determine whether NETs play any role in how dendritic cells, PMNs and macrophages acquire parasites and to examine whether this alters the outcome of *Leishmania* infection *in vivo*.

PAD4 is required for the citrullination of histones, which is an essential step in the formation of NETs. PAD4 deficient mice have been generated and used to study the function of NETs during microbial infection (Li *et al,* 2010), viral infection (Hemmers *et al,* 2011) and in a model of arthritis (Rohrbach *et al,* 2012).

Initially, in order to quantify the phagocytic capacity of different cell types *in vivo*, $1x10^6$ CFSE labelled *L. mexicana* promastigotes were i.d. injected into the ear dermis of $PAD4^{-/-}$ or C57BL/6 control mice. The ears were removed 24 h post-infection and split to form a dorsal and ventral sheets for explant culture (as described in Figure 6.1A). The recovered cells were counted and analysed using flow cytometric analysis to quantify the proportion and absolute number of macrophages ($F4/80^+$), DCs ($CD11c^+$) and neutrophils ($Ly6G^+$) relative to isotype control staining. Additionally, *Leishmania* uptake by each gated cell type was quantified, identifying cells that were $CFSE^+$ and therefore contain parasites or *Leishmania*-derived material. In this way it was possible to quantify the role of NETs in facilitating transfer of parasite-derived material to phagocytic cells during the first 24 h of infection.

In control C57BL/6 mice, macrophages and dendritic cells readily acquired parasites (or parasite-derived antigens) within 24 hours of infection with CFSE-labeled *L.*

mexicana. In contrast there were significantly fewer $CFSE^{+}$ neutrophils, macrophages and dendritic cells detected in the ear tissue of $PAD4^{-/-}$ mice (Figure 6.5A-C). This evidence suggests that the phagocytes are less able to acquire parasite material during the first day of infection in the absence of NETs. However, it should be noted that enumeration of total cell numbers in the ear tissue post infection revealed that there were significantly fewer immune cells recruited to the $PAD4^{-/-}$ ear dermis (Figure 6.5D) when compared with C57BL/6 mice. Overall, these data suggest that $PAD4^{-/-}$ mice failed to recruit immune cells to the site of infection as effectively as C57BL/6 mice and as a result fewer phagocytes were available to engulf whole parasites or parasite material. Taken together this evidence supports the idea that NETs are important for cell recruitment and for facilitating parasite uptake, at least in the early stages of infection.

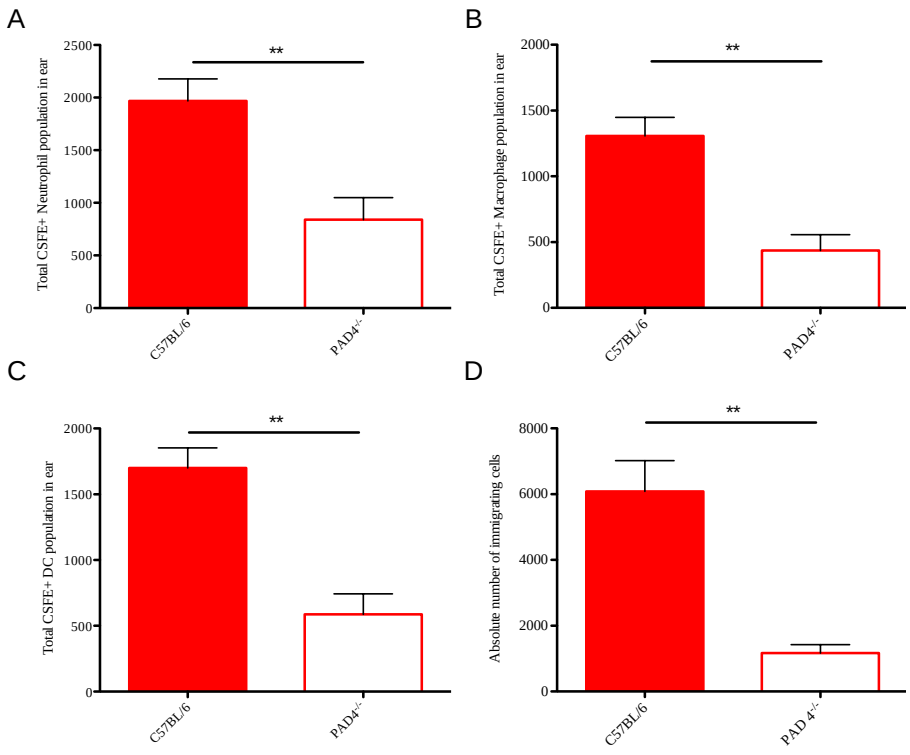


Figure 6.5. NET formation increases the acquisition of parasite-derived material by DCs and macrophages. The ears of PAD4$^{-/-}$ and C57BL/6 mice were infected with 1x10^{6} *L. mexicana* promastigotes. Ears were removed 24 hours later and cells recovered and analysed as described in Figure 6.1. Data show total number of CFSE^{+}Ly6G^{+} neutrophils (A), CFSE^{+}F4/80^{+} macrophages (B) or CFSE^{+}CD11c^{+} DCs (C) recovered from the explants. The graph represents the mean ± SEM of 3 biological replicates. Statistical analysis was performed using a Mann-Whitney U test. **=p≤0.01.

6.2.5. PAD4-deficient mice develop severe disease during promastigote initiated *Leishmania mexicana* infection

Having shown that a lack of NET formation in PAD4$^{-/-}$ mice results in reduced accumulation of phagocytes and subsequent impaired acquisition of parasite material

by phagocytes within the first 24 h of infection, the next experiments sought to investigate how this reduction in parasite uptake may affect disease progression in both promastigote and amastigote-initiated infections.

First, the hind-footpad of C57BL/6 and PAD4$^{-/-}$ mice were infected with 2.5x10^{6} *L. mexicana* metacyclic promastigotes by subcutaneous injection. The infected footpads were measured at weekly intervals over the course of infection, and the progression of disease was determined by calculating lesion size over time. Figure 6.6A and B depicts two separate experimental repeats in which lesion growth was significantly enhanced in PAD4$^{-/-}$ mice when compared with control C57BL/6 mice. Whilst the lesion was slow to develop in the first weeks of infection, it is clear that in the latter stages of infection there was a significant divergence in lesion size between mouse strains. Whereas C57BL/6 mice formed slow growing, small lesions over the infection period, PAD4$^{-/-}$ mice developed significantly larger lesions that increased rapidly from approximately 10 weeks post-infection (Figure 6.6). Post-mortem analysis of infected footpads also revealed significant differences in the parasite burden between C57BL/6 and PAD4$^{-/-}$ mouse strains. Limiting dilution assays were performed on the excised footpad lesions and, in both experiments, PAD4$^{-/-}$ mice showed significantly higher parasite burdens, than C57BL/6 mice **(**Figure 6.7A & B**)**. These data suggest that infection of PAD4$^{-/-}$ mice with *L. mexicana* promastigotes results in increased disease severity and suggest that NET formation is important for control of lesion formation and progression of disease.

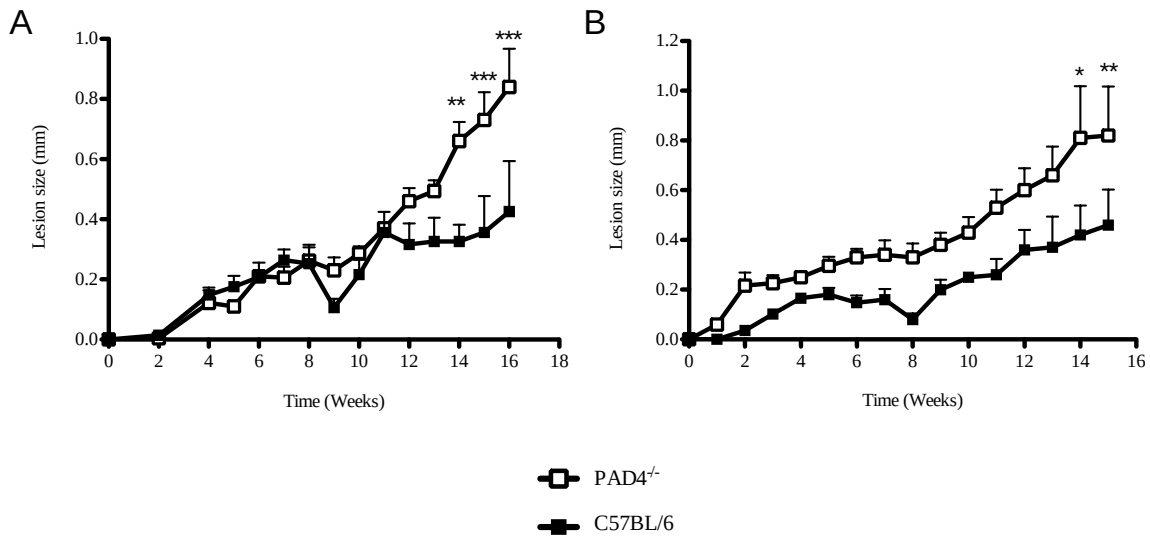


Figure 6.6. PAD4-deficient mice develop increased lesion growth upon infection with *Leishmania mexicana* promastigotes. C57BL/6 control (filled symbols) and PAD4$^{-/-}$ mice (empty symbols) were infected with 2.5x10^6 *L. mexicana* metacyclic promastigotes by s.c. injection into the hind footpad. The lesions were measured using spring-loaded calipers at weekly intervals over the course of infection. Results represent the mean ± SEM of 5 mice per group in two identical experiments (A & B). Statistical analysis performed using 2-way ANOVA with Bonferroni post-test. *=p≤0.05, **=p≤0.01, ***=p≤0.001. Statistical differences between PAD4$^{-/-}$ and C57BL/6 mice.

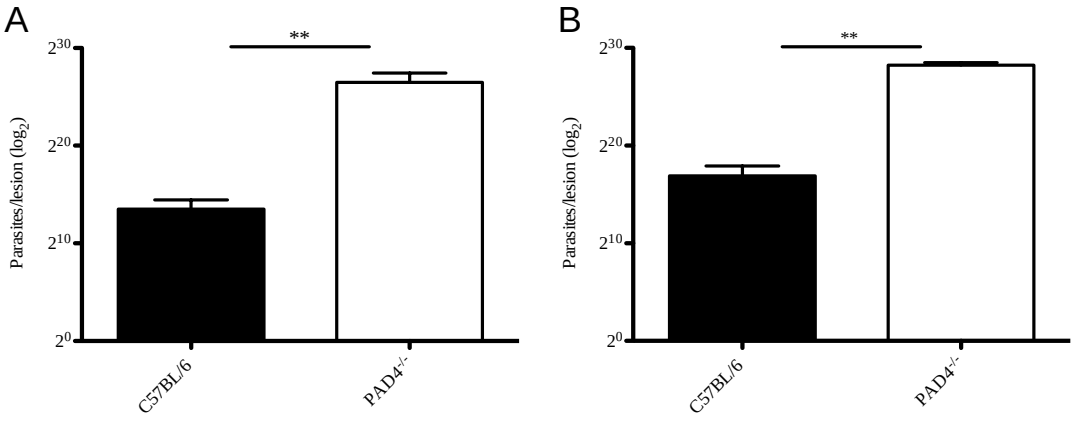


Figure 6.7. PAD4-deficient mice develop greater parasite burdens upon infection with *Leishmania mexicana* promastigotes. C57BL/6 and PAD4$^{-/-}$ mice were infected, as in Figure 6.6. The footpad lesion was excised, disrupted and the resulting cell suspension was used to determine the mean parasite burden by limiting dilution assay. Results represent the mean (± SEM) of 3 mice per group and show two identical experiments (A & B). Statistical analysis performed using and Mann-Whitney U Test. **=$p \leq 0.01$.

Haematoxylin and eosin staining (H&E) of excised lesions suggests that there was a high degree of cellular infiltrate in both C57BL/6 and PAD4$^{-/-}$ mice (Figure 6.8). However, the non-specific nature of H&E staining meant that it was not possible to accurately quantify individual cell types in the lesion. Therefore, IVIS imaging was performed at weekly intervals during infection to monitor neutrophil infiltration based on luminol bioluminescence associated with MPO activity (Figure 6.9). These data show that from 2 weeks post-infection there was a marginally higher influx of activated neutrophils detected in the footpad of PAD4$^{-/-}$ mice when compared with

C57BL/6 mice. Indeed, at later stages of infection this difference becomes more pronounced, with significantly greater total neutrophil influx in PAD4-deficient mice from 14 weeks onwards. These results demonstrate that the inability of PAD4$^{-/-}$ mice to generate NETs is associated with increased parasite burden and enhanced neutrophil influx at the site of *L. mexicana* infection, and that this becomes more pronounced as the lesion develops

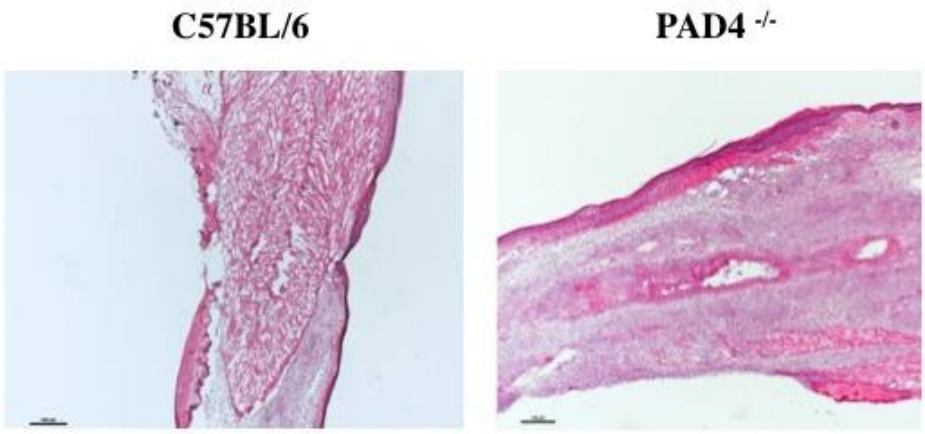


Figure 6.8. H&E histology of infected footpads suggests that PAD4^{-} deficient mice develop have a high degree of cellular infiltration at the site of *Leishmania mexicana* infection. Excised footpad lesions from mice in Figure 6.6 were frozen at -80 °C in OCT freezing medium and prepared for cryostat sectioning. 8 mm thick sections were stained with haemotoxylin and eosin stain (described fully in methods) and imaged using an upright Nikon (x 20 objective lens). Scale bar =100 μm.

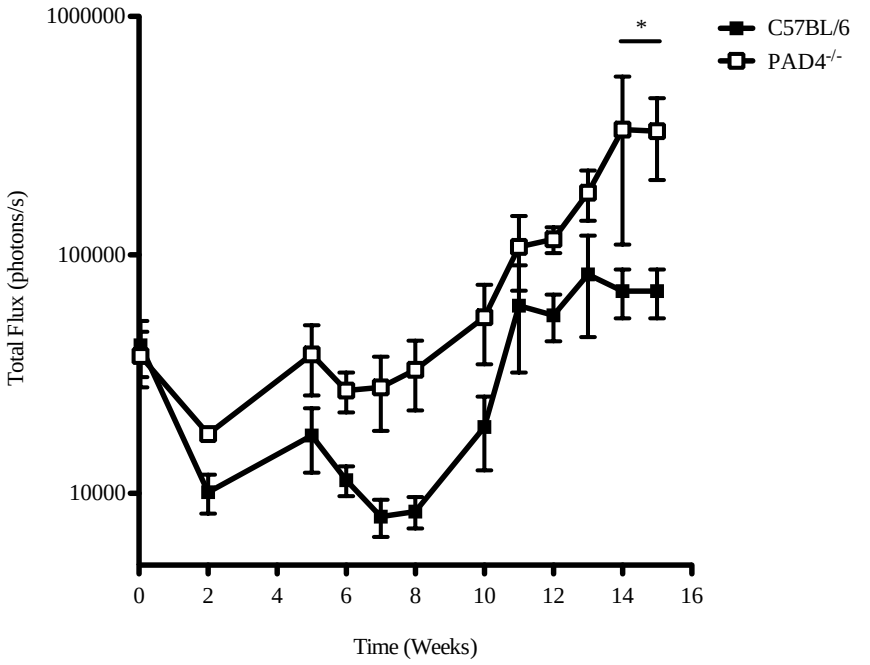


Figure 6.9. PAD4-deficient mice develop increased neutrophil influx at the site of *Leishmania mexicana* infection. C57BL/6 and PAD4$^{-/-}$ mice were infected, as in Figure 6.6 and at weekly intervals during infection neutrophil infiltration was quantified using IVIS imaging (as described in Figure 6.2.). Results represent the mean (± SEM) of 5 mice per group. Statistical analysis performed using 2-way ANOVA with Bonferroni pot-test. *= $p \leq 0.05$. Statistical differences are between C57BL/6 and PAD4$^{-/-}$ mice.

Having demonstrated that PAD4$^{-/-}$ mice develop more severe disease upon *Leishmania* infection, it was next important to determine how specific immune responses are affected by an inability to form PAD4-associated NETs. Firstly, serum antibody levels in *L. mexicana* infected C57BL/6 and PAD4$^{-/-}$ mice were quantified (Figure 6.10). Serum was tested by ELISA to quantify parasite-specific IgG1 and IgG2a and the reciprocal end-point dilution was used to determine the relative

antibody concentration in the sample. Interestingly, PAD4$^{-/-}$ mice had significantly higher IgG1 titres than C57BL/6 control mice in both experiments (Figure 6.10), suggestive of a more pronounced Th2-type antibody response in mice deficient in NET formation. It has been previously demonstrated that C57BL/6 mice will fail to produce significant levels of IgG2a during *L. mexicana* infection (Rosas *et al,* 2005) and in line with these findings negligible levels of IgG2a were detected in the serum of either C57BL/6 or PAD4$^{-/-}$ mice in both replicate experiments. In addition, the production of T cell-derived cytokines was quantified. From the infected C57BL/6 and PAD4$^{-/-}$ mice, splenocyte stimulation was performed using *L. mexicana* SLA and production of both IL-5 and IFN-γ quantified by ELISA. However, no significant differences in either IL-5 or IFN-γ concentrations were detected in either replicate experiments (Figure 6.11).

Taken together, these data suggest that the loss of PAD4 (and thus the ability to form NETs) results in significantly enhanced lesion growth following infection with *L. mexicana* promastigotes. The enhanced lesion size is associated with increased parasitaemia and enhanced neutrophil infiltration to the site of infection. Further to this, the loss of PAD4 enhances IgG1 antibody production, which suggest a skew towards the development of Th2 type immunity.

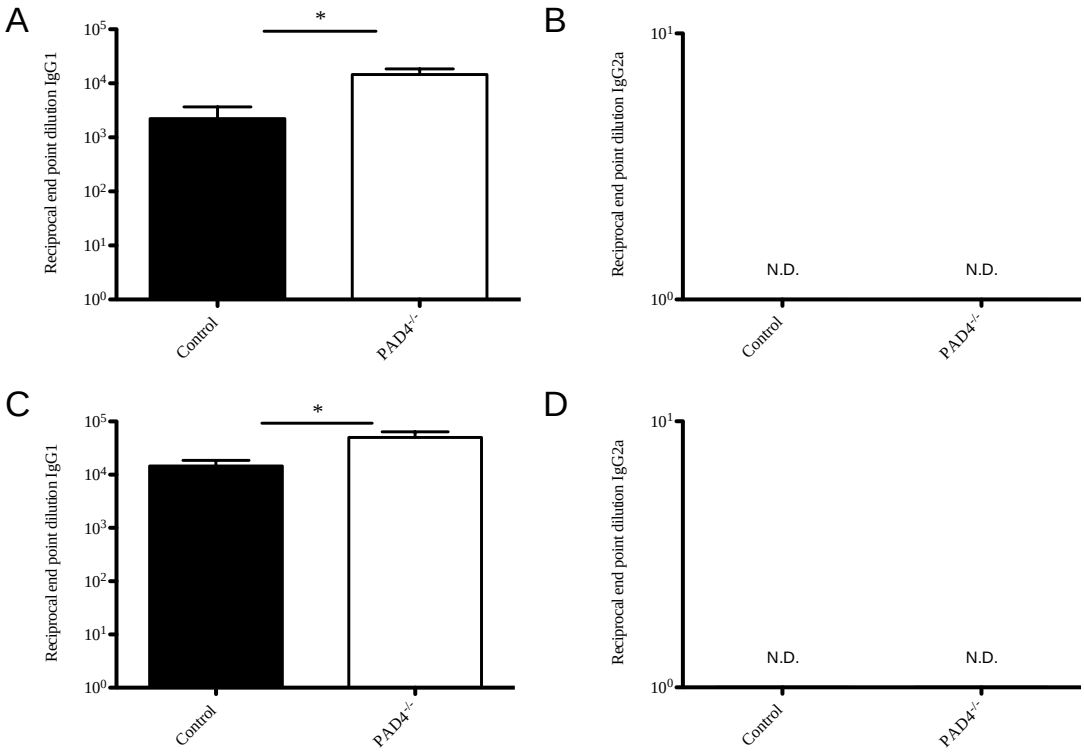


Figure 6.10. PAD4-deficient mice generate higher titres of parasite-specific IgG1 following *Leishmania mexicana* infection. Parasite-specific serum IgG1 and IgG2a levels of animals described in Figure 6.6 were quantified at 15 weeks post-infection using antibody ELISA. Results show the reciprocal end-point dilution and represent the mean (± SEM) of 5 mice per group in two identical experiments (A/B & C/D). N.D.=not detected. Statistical analysis performed using an Unpaired T-test *= $p \leq 0.05$.

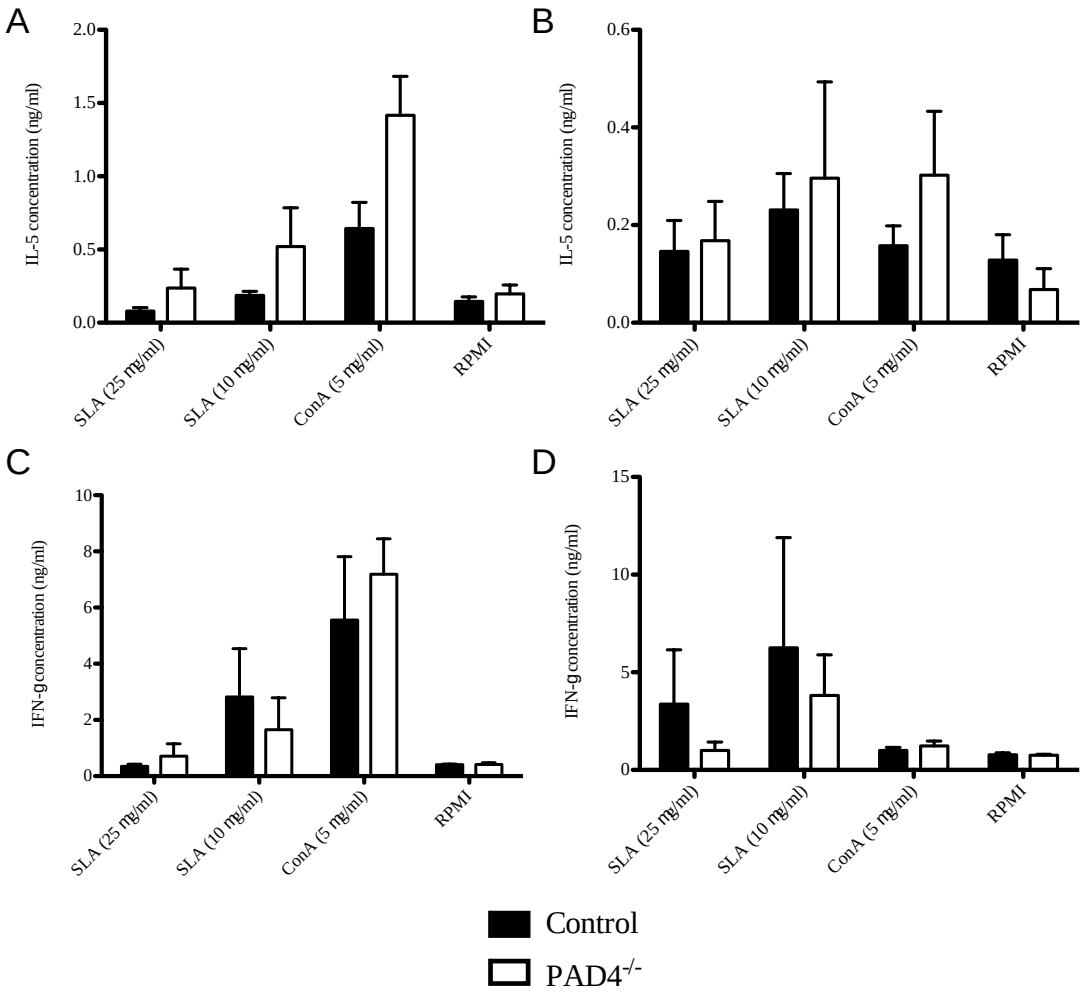


Figure 6.11. Splenocyte cytokine production in response to soluble *Leishmania mexicana* antigen. The spleens of *L. mexicana* infected C57BL/6 control mice and PAD4$^{-/-}$ mice were disrupted and the splenocyte suspension was stimulated with 25 μg/ml or 10 μg/ml of SLA (or with the mitogen, ConA). ELISAs were performed on supernatants to detect levels of IL-5 (A & B) and IFN-γ (C & D) in two identical experiments (A & C - Experiment 1; B & D – Experiment 2). Results represent the mean (± SEM) of 5 mice per group. Statistical analysis

performed using 2-way ANOVA with Bonferroni post-test. No significant differences were identified.

6.2.6. PAD4-deficient mice develop severe disease during amastigote initiated *Leishmania mexicana* infection

In support of previous reports that suggest a leishmaniacidal function for NETs (Guimaraes-Costa *et al,* 2009), the results above demonstrated that promastigote-initiated *Leishmania* infection of PAD4$^{-/-}$ mice results in an increased disease severity. However, it is not understood whether amastigotes can induce NETosis and whether the resulting NETs could effectively trap and kill the parasites. Further to this, it is unclear whether NETs are only important during initial infection with highly motile promastigotes, or if NETs have a function during the development of the lesion when parasites have matured to the intracellular amastigote form. Therefore, it was next investigated whether infection of PAD4-deficient mice initiated with amastigote parasites would have a similar outcome to promastigote infection.

Similar to the previous experiments, C57BL/6 and PAD4$^{-/-}$ mice were infected with *L. mexicana* amastigotes by subcutaneous injection into the hind footpad. As anticipated, following infection initiated by amastigotes lesion growth was rapid when compared with promastigote-induced infections (Figure 6.6). However, lesion development following amastigote infection followed a very similar trend to that observed during promastigote infection, with PAD4$^{-/-}$ mice developing significantly larger lesions than C57BL/6 mice from approximately 4 weeks post-infection until the infection end-point (Figure 6.12).

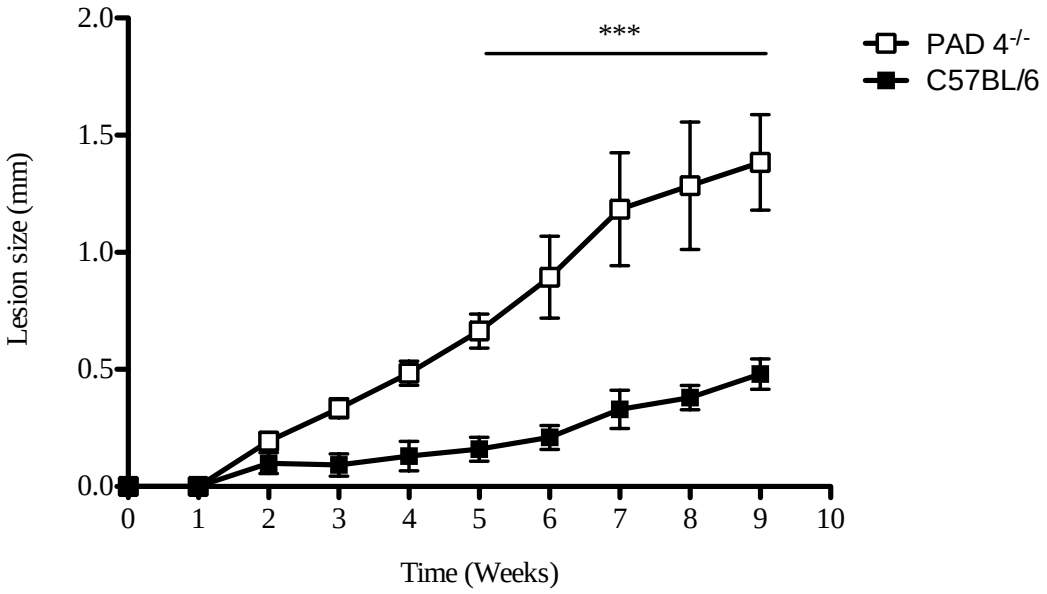


Figure 6.12. PAD4-deficient mice develop increased lesion growth upon infection with *Leishmania mexicana* amastigotes. C57BL/6 control (filled symbols) and PAD4$^{-/-}$ mice (empty symbols) were infected with 2.5x10^{6} *L. mexicana* amastigotes by s.c. injection into the hind footpad. The lesions were measured using spring-loaded calipers at weekly intervals over the course of infection. Results represent the mean (± SEM) of 5 mice per group. Statistical analysis performed using 2-way ANOVA with Bonferroni post-test. ***=$p \leq 0.001$. Statistical differences between C57BL/6 and PAD4$^{-/-}$ mice.

Next, the parasite burden in either mouse strain was determined. As observed in promastigote-induced infection, the lesions of PAD4$^{-/-}$ mice infected by amastigote parasites contained significantly more parasites than C57BL/6 mice (Figure 6.13). Additionally, as in the previous experiment with promastigotes, histological staining (H&E) of excised lesions suggests that following infection with amastigotes there

was a similarly enhanced level of cellular infiltrate in both C57BL/6 and PAD4$^{-/-}$ mice (data not shown).

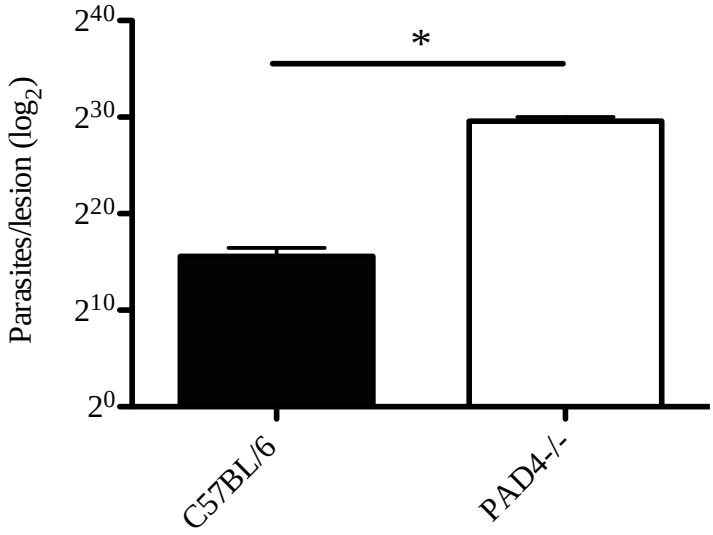


Figure 6.13. PAD4-deficient mice develop greater parasite burdens. C57BL/6 and PAD4$^{-/-}$ mice were infected, as in Figure 6.12. The footpad lesion was excised, disrupted and the resulting cell suspension was tested by limiting dilution assay to determine the mean parasite burden. Results represent the mean (± SEM) of 3 mice per group and show two identical experiments (A & B). Statistical analysis performed using a Mann-Whitney U Test *=p≤0.01. Statistical difference between C57BL/6 and PAD4$^{-/-}$ mice.

Similarly to mice in which infection was initiated with promastigotes, analysis of the immune response following amastigote-induced infection suggested that PAD4$^{-/-}$ mice produced significantly higher *Leishmania*-specific serum IgG1 than C57BL/6 mice (Figure 6.14). Again, negligible titres of *Leishmania*-specific IgG2a were found in both C57BL/6 and PAD4$^{-/-}$ mice. Futher to this, SLA specific T cell cytokine

analysis revealed that there were no significant differences between IL-5 and IFN-γ concentrations follwing stimulation with antigen between mouse strains (Figure 6.15).

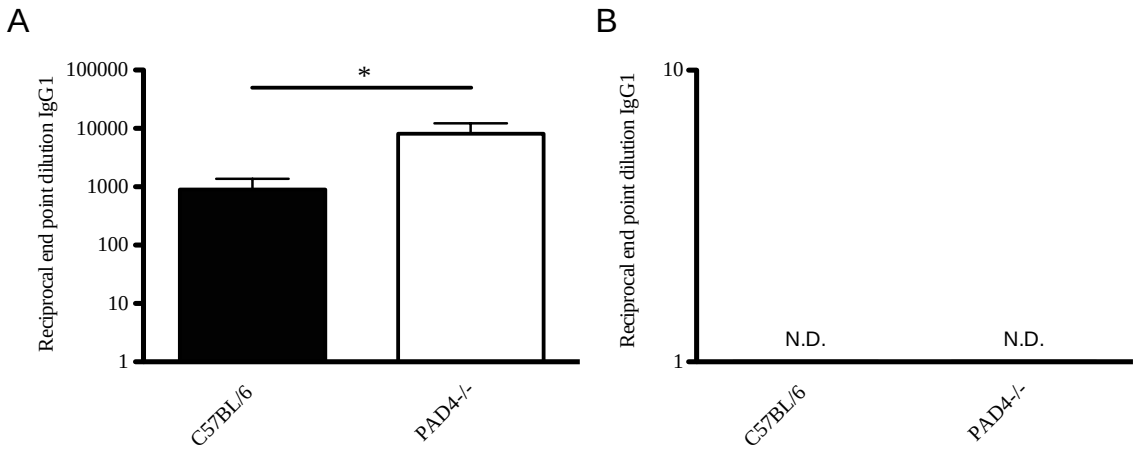


Figure 6.14. PAD4-deficient mice have a higher IgG1 titre, but produce no IgG2a. Parasite-specific serum A) IgG1 and B) IgG2a levels of animals described in Figure 6.12 were quantified at 9 weeks post-infection using antibody ELISA. Results show the reciprocal end-point dilution and represent the mean (± SEM) of 5 mice per group. N.D. = not detected. Statistical analysis performed using an Unpaired T-test. *=p≤0.01. Statistical difference between C57BL/6 and PAD4-/- mice.

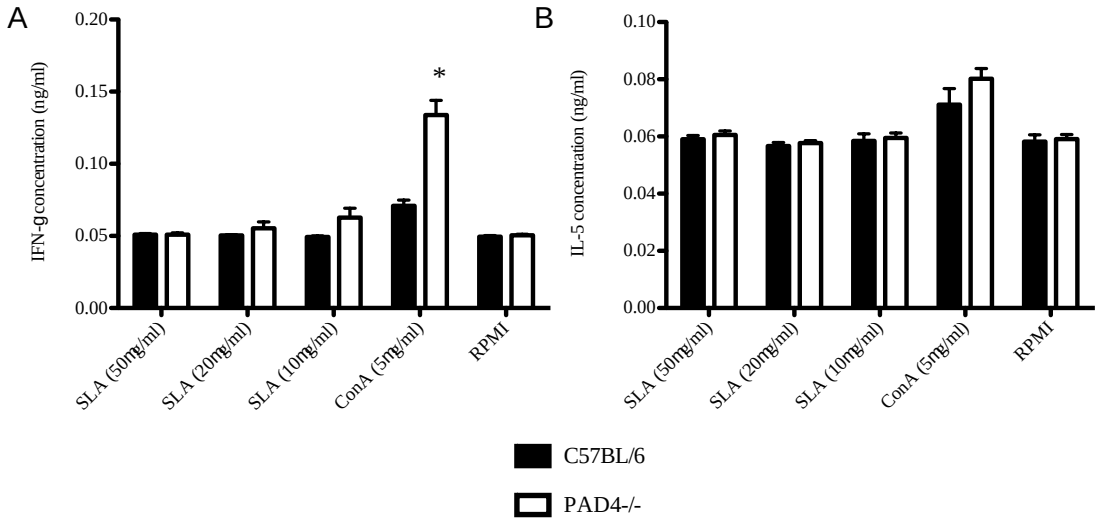


Figure 6.15. Splenocyte cytokine production in response to soluble *Leishmania mexicana* antigen. The spleens of *L. mexicana* infected C57BL/6 control mice and PAD4$^{-/-}$ mice were disrupted and the splenocyte suspension was stimulated with 25 μg/ml or 10 μg/ml of SLA (or with the mitogen, ConA). ELISAs were performed on supernatants to detect levels of IFN-γ (A) and IL-5 (B). Results represent the mean (± SEM) of 5 mice per group. Statistical analysis performed using a 2-way ANOVA with Bonferroni post-test. *=p≤0.01. Statistical difference between C57BL/6 and PAD4$^{-/-}$ mice.

These data demonstrate that amastigote-initiated infection of PAD4$^{-/-}$ mice proceeds in an almost identical manner to that observed following infection initiated with promastigotes. This evidence supports that the deletion of PAD4-derived NETs enhances lesion growth and parasite burden and promotes a Th2 type immune response.

These data have demonstrated that the ability to form NETs is important for immune cell recruitment, and uptake of promastigote material by phagocytic neutrophils, macrophages and DCs. The evidence suggests that this will have a profound effect on control of disease upon infection with both promastigote and amastigote forms of the parasite.

6.3. Discussion

In recent years there has been some controversy as to the prominence and function of neutrophils in *Leishmania* infection. Whilst, it has been widely demonstrated that neutrophils rapidly infiltrate infected tissue following *L. major* and *L. donovani* infection (McFarlane et al 2008; Ritter *et al,* 2009; Peters *et al,* 2008; Beil *et al*, 1992; Tacchini-Cottier *et al,* 2000), the exact kinetics of neutrophil migration and the ultimate role of neutrophils in the development of the adaptive immune response is not fully understood, particularly during the early stages of *L. mexicana* infection when the chronic disease state may become initiated. The data presented in this Chapter show that that neutrophils are efficiently recruited to the site of *L. mexicana* infection in BALB/c mice. Preliminary data, suggest that 24h after infection there is a marked increase in the number of neutrophils at the site of injection. A recent study by Hurrell *et al* (2015) has demonstrated similar results for *L. mexicana* infections in C57BL/6 mice. As such, it was anticipated that this early wave of neutrophil infiltration might influence the subsequent host response, as has been demonstrated in both *L. major* and *L. donovani* infection respectively (Tacchini-Cottier *et al,* 2000; McFarlane *et al,* 2008).

Consequently, it was demonstrated that NIMP-R14 mAb mediated depletion of the initial influx of migratory neutrophils during the first days of *L. mexicana* infection resulted in a significantly increased disease severity relative to BALB/c mice with an intact neutrophil compartment. Interestingly, others have identified neutrophils as possible host cells for the parasites, providing a niche for parasite development and evasion of host innate immune cell attack. Peters *et al*, (2010) and Ritter et al (2010) have demonstrated that neutrophils will rapidly engulf *L. major* parasites and retain

viable parasites, thus propagating further macrophage infection, and Laskay *et al* (2008) have proposed that neutrophils can help provide *L. major* a route of silent entry into macrophages during the first hours of infection. Therefore, it would be logical to assume that neutrophil depletion during this critical time of infection would reduce disease severity, rather than enhance it. However, Ritter *et al* (2010) have critically evaluated the proposed 'host-cell' role of the neutrophil during *Leishmania* infection and highlighted the importance of parasite strain and genetic background as a major factor in this matter. This is demonstrated by the studies of Tacchini-Cottier *et al* (2000) that have shown that while neutrophil depletion during the first days of *L. major* infection in BALB/c mice will reduce disease severity, neutrophil depletion of C57BL/6 mice will result in a transient exacerbation of disease. In contrast, while short-term monoclonal antibody depletion of neutrophils in BALB/c mice in the present study promoted *L. mexicana* infection, infection was controlled in neutropenic C57BL/6 mice (Hurrell *et al*, 2015). Additionally, depletion of neutrophils during the first weeks of *L. donovani* infection in BALB/c mice results in exacerbation of disease and, importantly, a skewed Th2 type immune response (McFarlane *et al*, 2008). Further to this, Novais *et al* (2009) suggest that depletion of neutrophils confers protection against *L. braziliensis* infection and even suggest a co-operation between macrophages and neutrophils, in a contact dependent mechanism for parasite killing. It is clear neutrophil depletion, transient or otherwise, during infection with visceral and cutaneous *Leishmania* species in mice of different genetic backgrounds has vastly differing outcomes.

Whilst neutrophils may aid in the dissemination and survival of *L. major* parasites (Peters *et al,* 2010; Laskay *et al,* 2008) in BALB/c mice, in the present study the

neutrophil appears to enhance disease severity during *L. mexicana* infection as depletion of the initial wave of neutrophils, by NIMP-R14 treatment, in BALB/c mice causes increased lesion growth. Charmoy *et al* (2010) have demonstrated that CCL3 expression in C57BL/6 mice is severely impaired post-NIMP-R14 treatment and that this reduces DC recruitment to the site of *L. major* infection. Conversely this group have shown that depleting neutrophils in C57BL/6 mice infected with *L. mexicana* increases DC recruitment to the site of infection and promotes healing and a Th1 response (Hurrell *et al*, 2015). As has been fully discussed in Chapter 3 a reduction in DC influx to the infection site, and subsequent reduction of antigen retrieval and presentation by these APCs, will impact the adaptive immune response.

The data presented in the present study, demonstrates that there was no observed increase in *L. mexicana*-exposed DC migration in the absence of neutrophils in these studies in either BALB/c or C57BL/6 mice. These findings contrast with the recent observations of Hurrell *et al* (2015) suggesting that, in Genista mice (which lack neutrophils (Ordonez-Rueda *et al,* 2012)), DC readily accumulate in the lymph node following *L. mexicana* infection and prime a Th1 response. However, the fact that neutrophils, during *L. mexicana* infection, are unable to induce DC mobilisation is in agreement with evidence suggesting that, unlike *L. major*, this parasite enters C57BL/6 neutrophils silently and does not induce CCL3 production (Hurrell *et al*, 2015).

Thus, it is clear that the neutrophil has a multi-faceted role during infection, and relatively recently, it has been demonstrated that neutrophils can also trap and kill pathogens via the extrusion of their DNA and the subsequent formation of NETS and a number of studies have demonstrated that NETs can affect the viability and

survival of bacteria, viruses and protozoa. In 2004, Brinkman *et al* visualised the interaction between *Shigella flexneri* and NETs and demonstrated that NETs were essential for the sequestration of the bacteria and that NET associated proteases, like neutrophil elastase could degrade *S. flexneri* virulence factors. In addition, the authors reported that histones found on NET fibres were capable of killing the bacteria *in situ*. Importantly, the reported microbicidal nature of NETs does not appear be limited to bacteria, and Guimares-Costa *et al*, (2009) have shown *in vitro* that *L. amazonensis* parasites are killed by specific histone arrangements in neutrophil DNA. Therefore it is of significance that *L. mexicana* infection in PAD4$^{-/-}$ mice on a C57BL/6 background results in increased disease severity, with a reduced capacity for NET formation resulting in significantly larger lesions, increased parasite burden and enhanced neutrophil infiltration to the site of infection relative to wild-type mice. Interestingly, this would correlate with neutrophil infiltration into the site of infection being associated with disease severity in C57BL/6 mice as reported by Hurrell *et al* (2015). While other studies have shown *L. donovani, L. major and L. infantum* can escape NET killing *in vitro* (Gabriel *et al* 2010; Guimares-Costa 2014), *L. mexicana* parasites belong to the same complex as *L. amazonensis* (which is susceptible to NET-mediated killing (Guimaraes-Costa *et al,* 2014)) and generally induce similar disease profiles in mice (McMahon-Pratt and Alexander 2004). What may be significant here is that Abdallah *et al* (2012) have recently demonstrated that NETs can affect the viability as well as the survival of *Toxoplasma gondii* tachyzoites following intranasal infection, and that surviving parasites have reduced infectivity. Thus NETs may reduce the viability rather than kill *L. mexicana*

promastigotes, and this would explain the increased parasite growth in their absence *in vivo* observed in the present study.

PAD4$^{-/-}$ mice have been previously used to study the role of NETs in arthritis (Rohrbach *et al,* 2012). In these studies, it has been shown that NETs are formed in the inflamed synovium of arthritis patients, and are present at least 5 days after initiation of inflammation. Thus, it is possible that during the chronic inflammatory response post *L. mexicana* infection, NET formation may occur during lesion development. If this is the case, NETs may contribute to the trapping of free amastigotes (post-macrophage rupture) and could possibly facilitate the killing of the intracellular form of the parasite. The results presented here using amastigote-initiated infections demonstrating enhanced parasite growth in PAD4$^{-/-}$ mice compared with wild-type counterparts would indicate this as a strong possibility.

Further to this, the data presented here suggest that in the absence of NETs, there is reduced macrophages and DCs acquisition of *L. mexicana* promastigote-derived material in the 24h following ear dermis inoculation, coupled with reduced cell recruitment in the early stages of infection. Although this work is preliminary and requires further investigation, these results support the hypothesis that NETs are important for the trapping and potential killing of free promastigotes following initial infection. Indeed, NETs have been shown to be important in the trapping of neutrophil associated autoantigens and appear to aid transfer of these antigens to myeloid DCs, a process essential for the development of associated autoimmune disease (Sangaletti *et al,* 2012). Therefore, it could be hypothesised that the immobilisation of parasites via NETs facilitates the uptake of parasite and parasite material by macrophages and DCs. Thus, in the absence of NETs, macrophages and

dendritic cells are not able to effectively acquire whole parasites or parasite fragments and there will be a downstream effect on parasite antigen presentation and the development of an adaptive immune response.

In summary, it is clear that neutrophils play an essential role in determining the outcome of *L. mexicana* infection. The data presented here suggest that neutrophils rapidly migrate to the site of infection following infection, and from the depletion studies it is apparent that this "early wave" of migratory neutrophils is important in controlling disease severity. Further to this, neutrophil depletion diminishes the migratory capacity of DCs towards and into the draining lymphatic vessels. Crucially, the protective role for neutrophils may depend on NETosis since infection of PAD4$^{-/-}$ deficient mice is associated with enhanced lesion growth and skewing of immunity towards a Th2 type response – both outcomes that may be implicitly linked to altered uptake and antigen presentation of parasites or parasite-derived material early in infection.

Chapter 7. The role of IL-4 and IL-4Rα in the progression of *Leishmania mexicana* infection

7.1. Introduction

As described in Chapter 1, a plethora of cytokines play a crucial role in determining the outcome of cutaneous *Leishmania* infection and the balance of protection versus chronicity is often associated with the comparative Th1/Th2 bias (Reiner and Locksley, 1995; Alexander *et al,* 2002; Rosas *et al,* 2005). Mice deficient in IL-12 or IFN-γ are rendered susceptible to *Leishmania major* infection, even when on C57BL/6 or 129 backgrounds which would normally be resistant (Mattner *et al,* 1996; Wang *et al,* 1994). Conversely, mouse strains normally susceptible to *L. mexicana* either deficient in IL-4 (Satoskar *et al,* 1995) or lacking IL-4Rα, (a component of the receptor for both IL-4 and IL-13) are resistant to *L. mexicana* (Alexander *et al,* 2002), although IL-4Rα$^{-/-}$ mice still develop a Th2 response following infection (Mohrs *et al,* 2000). However, there are multiple cell sources of IL-4 as well as many cellular targets and interestingly IL-4Ra$^{-/-}$ mice on a (BALB/c background) are more susceptible than IL-4$^{-/-}$ mice to *L. major* infection indicating that some cells responding to IL-4 may play a role in protection. Indeed it has been shown that the signature Th2 cytokines IL-4/IL13 can, under certain conditions, facilitate a Th1 response in *Leishmania* infections (Biedermann *et al* 2001; McFarlane *et al*, 2011).

Together, these reports highlight the importance of cytokines in generating Th1 and Th2 responses, but the complex role of each of these cytokines remains unclear. In an attempt to delineate the role of these cytokines in infection, mice lacking IL-4Rα only on specific cell types have been generated. Mice in which the *IL-4Rα* gene is flanked by loxP sites have been crossed with a number of strains expressing Cre-recombinase under different cell-specific promoters including LysM^{cre}

(macrophages/neutrophils) (Radwanska *et al,* 2007), CD11c^{cre} (DCs) (Hurdayal *et al,* 2013), mb-1^{cre} (B cells) (Hoving *et al,* 2012) and Lck^{cre} (T cells) (Bryson *et al,* 2011) to create these cell-specific IL-4Rα-deficient animals. Such studies have suggested that IL-4/IL-13 signaling in T cells is important in the progression of *L. mexicana* and *L. major*, although the role for IL-4 in the early stages of *Leishmania* infection remain ill-defined (Bryson *et al,* 2010).

Crucially, the work of Biedermann *et al* (2001) and Ehrchen *et al* (2010) suggest that early IL-4 has an impact on DC function and is important in the development of protective Th1 type immunity in *L. major* infection. The results presented in Chapter 3 demonstrate that DC migration to the draining LN (dLN) is abrogated during *Leishmania mexicana* infection, and therefore the primary aim of this chapter was to determine whether IL-4 may play a role in influencing the DC response following infection. Mice in which only DCs lack IL4-Rα were used to determine how DCs migration is altered by IL-4 and to assess the resulting outcome on *L. mexicana* infection and associated immune responses in the absence of IL-4/IL-13 responsive DCs.

As discussed in Chapter 1, the role of IL-4 in susceptibility to *Leishmania mexicana* infection has been hypothesised to be associated with the generation of IgG1/FcγRIII-dependent induction of IL-10 (Thomas and Buxbaum, 2008). B cell IgG1 production is primarily dependent on B cells responding to IL-4 (Snapper *et al* 1988). During the course of my studies, mice in which only the B cell compartment lacks IL-4Rα became available (Hoving *et al,* 2012). Following immunisation of with a model antigen, isotype switching in these B cell-specific IL-4Rα$^{-/lox}$ animals is

skewed away from IgG1 production and towards IgG2a, providing the capacity to uncouple Th2 responses from the consequences of IgG1 signaling (Hoving *et al,* 2012). Therefore, the secondary aim of this chapter was to examine the consequences of IL-4Rα-depletion upon the development of *L. mexicana* infection in an attempt to define the function of IgG1 in disease progression.

7.2. Results

7.2.1. BALB/c mice lacking IL-4Rα specifically on DCs develop progressive disease during *Leishmania mexicana* infection.

As highlighted above, production of IL-4 early in *L. major* infection appears to play an important role in the optimal generation of a protective Th1 response by acting upon DCs to stimulate IL-12 (Beidermann *et al,* 2001: Yao *et al,* 2005; Ehrchen *et al*, 2010). However, from these previous studies in which mice were either treated with exogenous IL-4 (Beidermann *et al,* 2001) or with a blocking antibody (Ehrchen *et al*, 2010) soon after infection, an indirect effect of IL-4 on non-DCs cannot be excluded.

During the course of this project, a strain of mice lacking expression of IL-4Ra specifically on DCs ($CD11c^{cre}$IL-$4R\alpha^{-/lox}$) was generated in Prof F. Brombachers' laborarory (Hurdayal *et al*, 2013). These mice were created by back-crossing $CD11c^{cre}$ mice with IL-$4R\alpha^{-/-}$ animals, and then breeding the resulting $CD11c^{cre}$IL4$Ra^{-/lox}$ with IL-$4R\alpha^{lox/lox}$ mice to generate $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ animals in which the floxed *IL-4Rα* gene was removed only in cre recombinase-expressing $CD11c^{+}$ cells. Rump infections of littermate controls, globally IL-4Rα-deficient, and $CD11c^{cre}$IL-$4R\alpha^{-/lox}$ (DC-specific IL-4Rα-deficient) were carried out by injecting $5x10^{6}$ *L. mexicana* amastigotes into the base of the tail and lesion growth was monitored during infection as a measure of disease progression.

Unexpectedly, it was found that lesion growth was rapid in all of the groups. In fact, early in infection (2-3 weeks) lesions were uncharacteristically large relative to other experiments (eg. Figure 7.1). Post-experimental autopsy suggested that the mice had

contracted a secondary co-infection, which could explain the observed exacerbation of disease. However, although the experiment was somewhat compromised for this reason, it can be noted that both IL-4Rα$^{-/lox}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice developed large progressive lesions, whilst IL-4Rα$^{-/-}$ mice had smaller lesions that were reducing in size over time (Figure 7.1A).

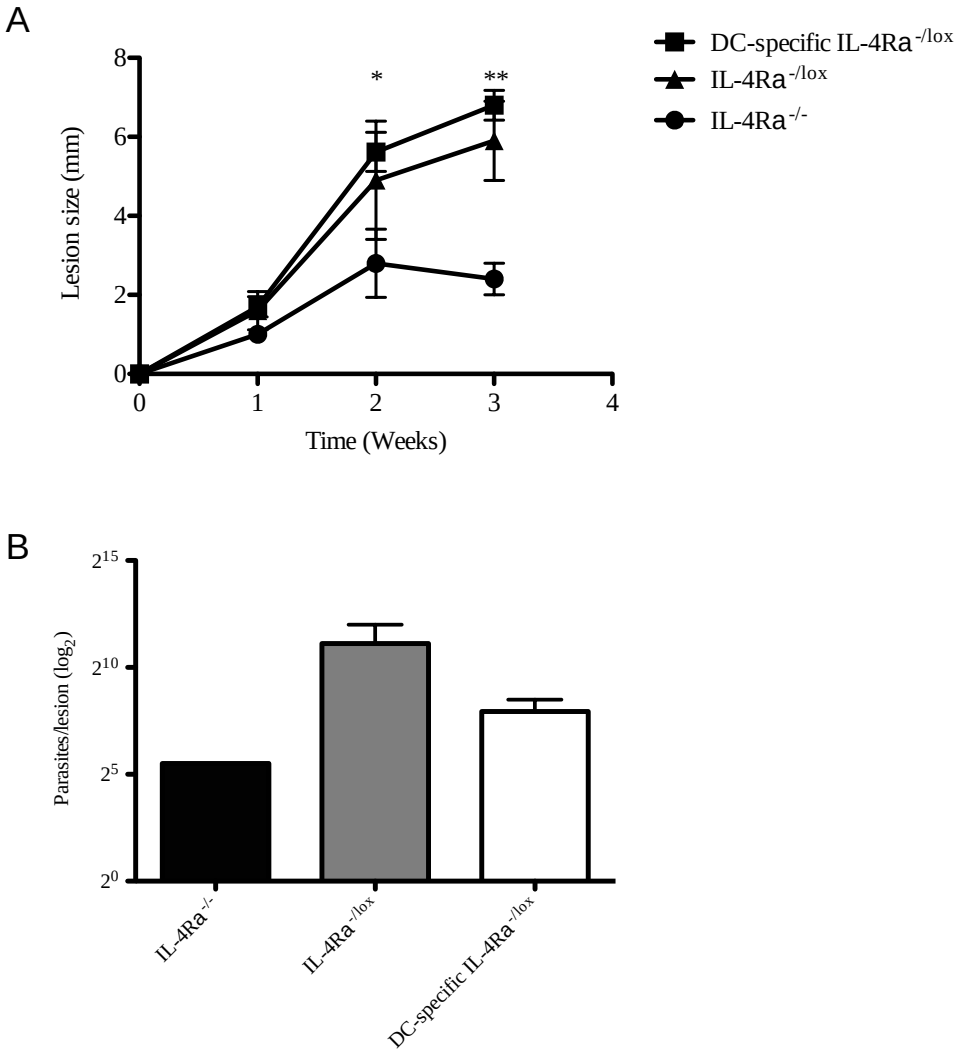


Figure 7.1. Lesion growth and parasite burden following *Leishmania mexicana* infection is unaffected by DC-specific IL-4Rα deficiency. 5x10^6 *L. mexicana* amastigotes were injected s.c. into the shaven rump of

IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice and the lesions were measured at weekly intervals over the course of infection. Results (A) show the mean ± SEM of 5 mice per group and are representative of 2 experiments (one rump infection and an ear pinna infection). B) Mean parasite burdens in the footpad were determined by a limiting dilution assay. Results represent the mean ± SEM of 3 mice per group. Statistical analysis performed using 1-way ANOVA and Bonferroni post-test. *= $p<0.05$ **=$p<0.001$. Significance differences identified between footpad sizes of IL-4Rα$^{-/-}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice at 2 and 3 weeks post infection.

Furthermore, as shown in Figure 7.1A the lesions of IL-4Rα$^{-/-}$ mice contained fewer parasites than those of the wild-type animals, and DC-specific IL-4Rα-deficient mice showed no impairment to parasite growth (Figure 7.1B). However, it should be noted that the parasite numbers in all three groups were strikingly low and no significant differences were discernable. Therefore, these preliminary data suggest that IL-4Rα deletion specifically in DCs does not provide protection from early pathology or parasite growth. Despite the rapid lesion growth exhibited, the lower parasite burdens suggest that a component of this inflammation may be associated with a secondary infection in these animals or another confounding factor and, as such, these results should be assessed with caution.

To further investigate the difference between global IL-4Rα-deficient and DC-specific IL-4Rα-deficient animals, the cytokine production and parasite-specific

antibodies were quantified. No significant differences in the production of Th1 (Figure 7.2) or Th2 (Figure 7.2B) cytokines by splenocyte cultures from infected IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice were detected.

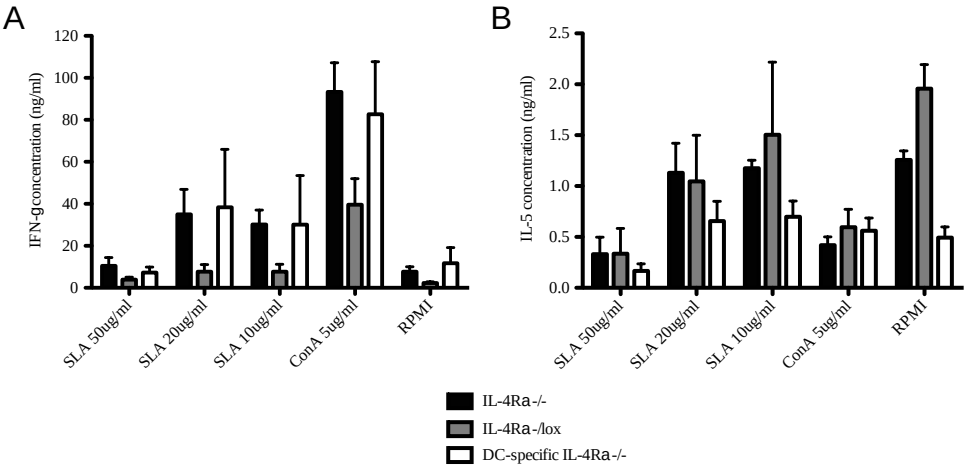


Figure 7.2. Splenocyte cytokine production in response to soluble *Leishmania mexicana* antigen (SLA). IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice were infected as described in Figure 7.1. The spleens were disrupted and the splenocyte suspension was stimulated with 50, 20, 10μg/ml of SLA. ELISAs were performed on supernatants to detect levels of A) IFN-γ and B) IL-5. Results show the mean ± SEM of 5 mice. Statistical analysis performed using 2-way ANOVA with Bonferroni post-test. No significant differences were identified.

As in previous studies (Hurdayal *et al,* 2012), *Leishmania*-specific IgG1 production was impaired in IL-4Rα$^{-/-}$ mice, but the titres were significantly higher in CD11c^{cre}IL-4Rα$^{-/lox}$ mice, similar to the levels detected in serum isolated from IL-4Rα$^{-/lox}$ control mice (Figure 7.3A). IgG2a levels were comparable in IL-4Rα$^{-/lox}$, IL-

4Rα$^{-/-}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice (Figure 7.3B).

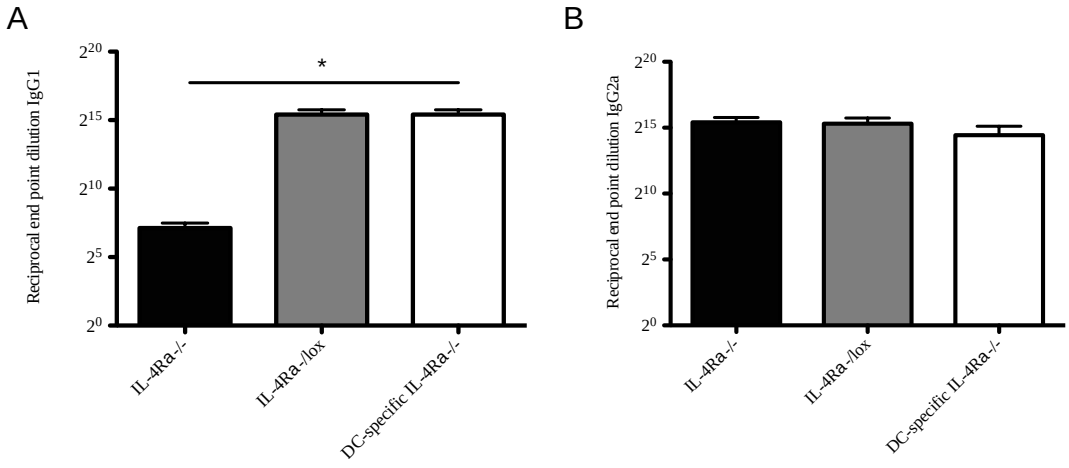


Figure 7.3. *Leishmania mexicana* specific antibody responses in DC-specific IL-4Rα deficient mice. IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice were infected as described in Figure 7.1. 3 weeks post-infection, the titre of SLA-specific IgG1 (A) and IgG2a (B) were established using ELISA. Results show the mean ± SEM of 5 mice per group. Statistical analysis performed using 2-way ANOVA with Bonferroni post-test. *=$p<0.05$ significant differences were identified between IL-4Rα$^{-/-}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice.

Together, these results show that similar to *L. major* infection (Hurdayal *et al,* 2014), *L. mexicana* infected CD11c^{cre}IL-4Rα$^{-/lox}$ mice develop non-healing lesions, and generate parasite-specific IgG1 responses higher than the global IL-4Rα-deficient animals. Whilst the IL-4Rα$^{-/-}$ mice are protected from infection through their ability to generate a robust Th1 response (Satoskar *et al*, 1995; Alexander *et al,* 2002), mice lacking the receptor only on DCs appear to be rendered susceptible to *L. mexicana* infection. This may fit with the model proposed above, in which early IL-4 plays a

crucial role in determining DC function, and is similar to the outcome of *L. major* infection (Hurdayal *et al,* 2013), however the mechanisms remain undefined. Given that the results in Chapter 3 demonstrate the important role of DC migration in immunity to *L. mexicana*, it was next investigated whether this early IL-4-driven response may impact upon DC function.

7.2.2. DC migration *in vivo* is dependant on DC responsiveness to IL-4/IL-13

The results above suggest that DC–specific IL-4Rα-deficient mice show a progressive disease phenotype following *L. mexicana* infection, and previous reports suggest that early IL-4 may influence DC function *in vivo* (Biedermann *et al,* 2001; Ehrchen *et al,* 2010). Therefore, the migratory capacity of bone marrow derived IL-4Rα$^{-/-}$ DCs was investigated *in vivo* using an approach similar to that applied in Chapter 3. First, bmDCs were generated from either wild-type (WT) or global IL-4Rα knockout mice. Once matured, DCs were CFSE labeled and transferred into the footpads of wild-type BALB/c recipient mice, ensuring that only DCs were compromised in their ability to respond to IL-4 and all other components were normal. To ensure that DCs were exposed to any potential source of early IL-4 it was necessary to co-inject LPS or parasites directly into the site of DC transfer, rather than to pre-treat the DCs *in vitro*. Therefore, either LPS (1 μg) or *L. mexicana* promastigotes ($2.5x10^6$) were injected into the footpad immediately after DCs transfer. After 18h the draining popliteal lymph node was removed, cells harvested and counted and subsequently prepared for analysis by flow cytometry. CD11c$^+$ DCs were identified and the proportion of CFSE$^+$ cells determined to calculate the total number of CFSE$^+$ DCs in the draining lymph node. As previously observed, whilst LPS stimulation resulted in efficient migration of CFSE-labeled DCs from the site of

inoculation to the draining LN, *L. mexicana* infection reduced this accumulation with fewer transferred wild-type DC detected in the lymph node (Figure 7.4). Interestingly, following LPS stimulation significantly fewer IL-4Rα$^{-/-}$ DCs migrate to the draining lymph node than wild type (WT) BALB/c DCs. However, it should be noted that following *L. mexicana* infection, there is no significant inhibition of the migratory capacity of IL-4Rα $^{-/-}$ when compared to WT DCs. These results suggest that IL-4/IL-13 may have a role in directing the functional migration of DCs under inflammatory conditions to the draining LN. However, this preliminary study does not provide convincing evidence to suggest that this is also the case during *L. mexicana* infection.

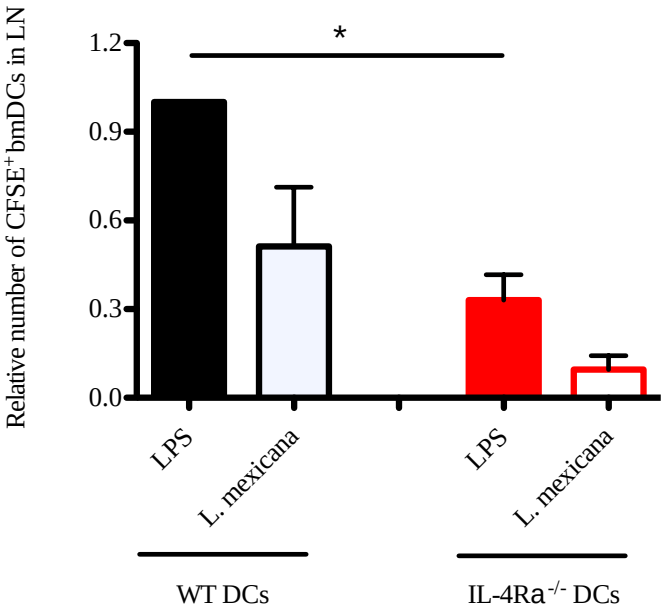


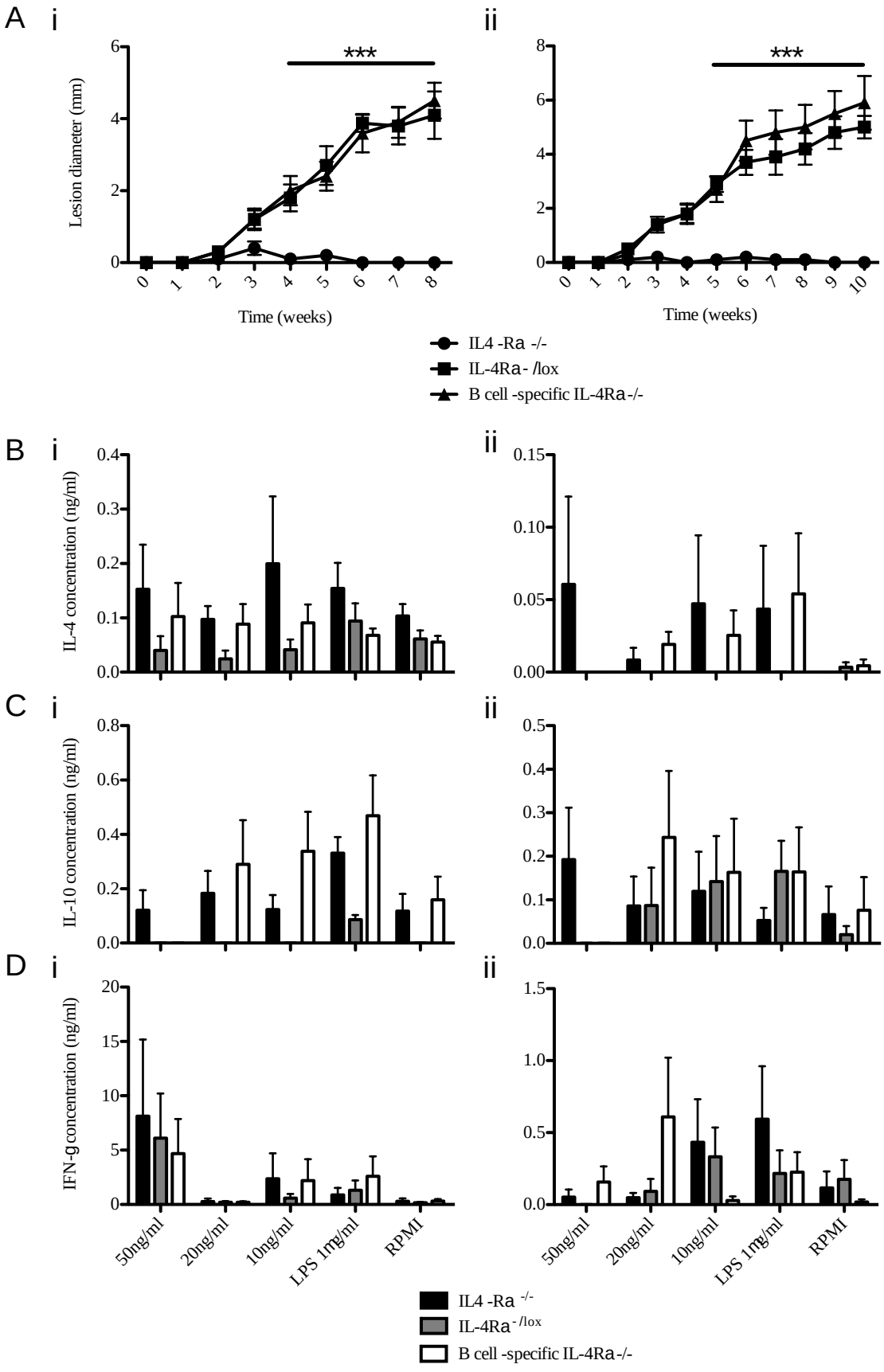
Figure 7.4. BmDCs lacking IL-4Rα show a failure to migrate to the dLN *in vivo*. Wild-type (WT) and IL-4Rα$^{-/-}$ bmDCs were CFSE labeled and injected into the footpads of BALB/c recipients. Simultaneously, LPS (1μg) or 2.5x10^6 *L. mexicana* promastigotes were injected into the

same footpad. 18 h post-transfer, draining popliteal LNs were removed, analysed by flow cytometry and the absolute number of $CD11c^{+}CFSE^{+}$ cells quantified. The graph shows the mean ± SEM of 3 mice per group normalised to the LPS WT value. Statistical analysis performed using one-way ANOVA with Bonferroni post test. *= $p<0.05$. Significant differences were identified between LPS treated WT and LPS treated IL-$4R\alpha^{-/-}$ mice.

7.2.3. BALB/c mice lacking the IL-4Rα-expressing B cells develop progressive disease during *Leishmania mexicana* infection, despite an absence of IgG1 antibody.

Given that recent reports have suggested that the non-healing response to *L. mexicana* is IgG1 dependent (Thomas and Buxbaum, 2008; Chu *et al,* 2010) and that IgG1 production is largely dependent upon the action of IL-4 production upon B cells, the secondary aim of this chapter was to examine the outcome of infection in mice lacking IL-4Rα only on B cells. $Mb1^{cre}$IL-$4R\alpha^{-/lox}$ mice were generated as previously described (Hoving *et al*, 2012). Briefly, mice were produced by back-crossing $Mb1^{cre}$ mice with IL-$4R\alpha^{-/-}$ (globally deficient) animals, and then breeding the resulting $Mb1^{cre}$IL-$4R\alpha^{-/-}$ with IL-$4R\alpha^{lox/lox}$ mice to generate $Mb1^{cre}$IL-$4R\alpha^{-/lox}$ (B cell-specific IL-4Rα-deficient) animals in which the floxed *IL-4Rα* gene was removed only in cre recombinase-expressing $Mb1^{+}$ cells. Cre negative mice, IL-$4R\alpha^{-/lox}$ (littermate control) mice expressing functional IL-4Rα were used as controls throughout. *L. mexicana* amastigotes were injected subcutaneously into the rump of IL-$4R\alpha^{-/lox}$, IL-$4R\alpha^{-/-}$ and $mb1^{cre}$IL-$4R\alpha^{-/lox}$ mice. Lesion growth was measured over the following weeks to monitor disease progression.

As previously demonstrated, whilst wild-type mice developed progressive, non-healing lesions, global IL-4Rα$^{-/-}$ mice were protected and failed to develop any significant infection. Interestingly, the B cell-specific IL-4Rα-deficient (mb1^{cre}IL-4Rα$^{-/lox}$) mice formed large, non-healing rump lesions similar to those seen in littermate control mice (Figure 7.5Ai and ii). Counter to the original hypothesis, these data suggest that abrogation of IL-4Rα signaling in B cells does not affect the development of the chronic non-healing lesion associated with *L. mexicana* infection. Unfortunately, the very low levels of cytokine detected in the supernatant of re-stimulated splenocytes may suggest that splenocytes failed to produce sufficient levels of cytokine during *ex vivo* incubation, with no significant differences detected in the levels of IL-4, IL-10 or IFNγ in either experiment (Figure 7.5B, C and D)


A
i
ii
***
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Lesion diameter (mm)
Time (weeks)
Time (weeks)
IL4 -Ra -/-
IL-4Ra- /lox
B cell -specific IL-4Ra-/-
B
i
ii
IL-4 concentration (ng/ml)
C
i
ii
IL-10 concentration (ng/ml)
D
i
ii
IFN-g concentration (ng/ml)
50ng/ml
20ng/ml
10ng/ml
LPS 1mg/ml
RPMI
IL4 -Ra -/-
IL-4Ra -/lox
B cell -specific IL-4Ra-/-

Figure 7.5. Rump lesion growth and associated T cell cytokine production following *Leishmania mexicana* infection is unaffected by B cell specific IL-4Rα deficiency. $5x10^6$ *L. mexicana* amastigotes were subcutaneously injected into the shaven rump of IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and mb1creIL-4Rα$^{-/lox}$ mice and the lesions were measured at weekly intervals over the course of infection. Results show the mean ± SEM of 5 mice per group in two separate experiments (A i and ii). The spleens were disrupted and the splenocyte suspension was stimulated with 50, 20, 10μg/ml of SLA. ELISA were performed on supernatants to detect levels of B) IL-4 C) IL-10 and D) IFN-γ. The figure shows results of two separate experiments i) and ii). Results show the mean ± SEM of 5 mice. Statistical analysis performed using 2-way and 1-way ANOVA for A) and B, C and D) respectively. Significant differences in footpad size were identified between IL-4Rα$^{-/-}$ and both IL-4Rα$^{-/lox}$ and CD11c^{cre}IL-4Rα$^{-/lox}$ mice from 4 weeks onwards in Ai and Aii. No significant differences were identified in cytokine levels.

As described above, IgG1 has been suggested to play a key role in the pathogenicity of *L. mexicana* infection (Chu et al, 2010). However, the data above suggested that mice in which B cells were unresponsive to IL-4 developed chronic, non-healing lesions upon infection. Previous studies using the B cell-specific IL-4Rα-deficient (mb1^{cre}IL-4Rα$^{-/lox}$) mice have shown that IgG1 production was impaired, and that antibody response was biased towards a Th1 type response, with elevated IgG2a levels post-OVA immunisation (Hoving *et al*, 2012). Therefore, antibody levels in

the serum of infected littermate control mice (IL-4Rα$^{-/lox}$), global IL-4Rα-deficient (IL-4Rα$^{-/-}$), and B cell-specific IL-4Rα-deficient mice were quantified 10 weeks after infection. It was found *L. mexicana* infection of either global or B cell-specific IL-4Rα-deficient mice failed to induce parasite-specific IgG1, whilst littermate control mice had a significantly higher titre of *Leishmania* antigen-specific IgG1 in serum (Figure 7.6A).

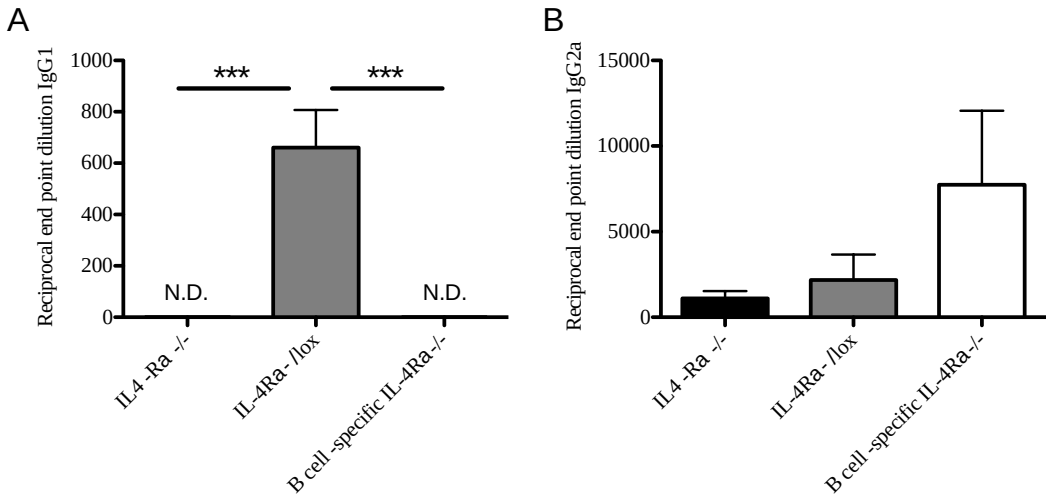

Figure 7.6. B cell specific IL-4Rα deficiency marginally enhances a Th1 type antibody response. IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and mb1^{cre}IL-4Rα$^{-/lox}$ mice were infected as described in Figure 7.5. 10 weeks post-infection, the titre of SLA-specific IgG1 (A) and IgG2a (B) were established using ELISA. N.D. = Not detected. Results show the mean ± SEM of 5 mice per group. Statistical analysis performed using 1-way ANOVA for. Significant differences were identified between IL-4Rα$^{-/lox}$, IL-4Rα$^{-/-}$ and mb1^{cre}IL-4R$^{α-/lox}$ mice.

Conversely, it was found that the level of soluble *Leishmania* antigen-specific IgG2a

was slightly (although not significantly) increased in B cell-specific IL-4Rα-deficient mice in comparison with levels detected in littermate control and global IL-4Rα-deficient mice (Figure 7.6). Together these data demonstrate suggest that B cell expression of IL-4Rα appears to be essential for generating IgG1 antibody production during *L. mexicana* infection. However, somewhat surprisingly, lesion growth appears to be unaffected by the loss of this receptor on B cells. Therefore, it would seem that a failure to produce parasite-specific IgG1 antibodies does not directly influence the progression of pathology at the site of *L. mexicana* infection in BALB/c mice.

7.3. Discussion

A variety of studies have demonstrated how IL-4Rα, and its cognate ligands IL-4 and IL-13, can directly and indirectly influence the function of immune cells (reviewed by Choi and Rieser, 1998). These cytokines have been identified as important mediators for the development of Th2 type helper immunity (Kopf *et al,* 1993) against a variety of pathogens and immune challenges; from nematodes (Else *et al,* 1994) and protozoan infection to autoimmune disease (Grunig *et al,* 1998; Chatelain *et al,* 1992).

Th2-associated cytokines and resultant immunity is synonymous with exacerbation of cutaneous *Leishmania* infection. It was Heinzel *et al* (1989) that originally associated the Th2 adaptive response with susceptibility to *L. major* infection. However, more recent evidence puts into question the function of canonical Th2 type cytokines, IL-4/IL-13 and queries whether, in different species, host genetic backgrounds, and even sites of infection, these cytokines can have a role in both susceptibility and resistance (Alexander and McMahon-Pratt, 2004; Alexander and Brombacher, 2012).

As has been discussed, IL-4 will impact the development of T helper immunity, but will also direct other arms of the immune response, namely B cell development (Finklemann *et al,* 1989). Th2 and/or Tfh-derived IL-4 (Reinhardt *et al,* 2011) have been shown to be important in the activation of B cells, differentiation of B cells into plasma cells and switching to production of IgG1 (Snapper *et al,* 1988). Thus, the creation of cell-specific IL-4Rα knock out mice has been advantageous for dissecting the function of IL-4Rα following *L. mexicana* infection. Hoving *et al*

(2012), used a model of colitis to demonstrate a role of IL-4/IL-13 in development of chronic inflammation. The mice were challenged with OVA and it was found that IgG1 and IgE antibody production was significantly abrogated whilst IgG2a levels were enhanced (Hoving *et al,* 2012). In addition, when colitis was induced by oxazalone treatment in the mice, the animals failed to develop disease. Similarly, the results presented here demonstrate that B cell responsiveness to IL-4/IL-13 is necessary for the production of IgG1, with parasite-specific IgG1 production completely abrogated following *L. mexicana* infection of mice lacking IL-4Rα-expressing B cells.

Finkleman *et al* (1988) initially demonstrated that IL-4 is required for IgE and IgG1 production, and further work by Kane and Mosser (2002) implicated IgG1 as an essential mediator of IL-10 production, primarily from macrophages following stimulation of FcγRIII. Further to this, Chu *et al* (2010) suggested that, by inducing IL-10 production in this way, IgG1 is detrimental to the development of a healing response in *L. mexicana* infection. Therefore it was hypothesised that a reduction in IgG1 production in mice lacking IL-4Rα-expressing B cells would diminish pathogenesis. The results presented here are somewhat contradictory to this assumption – whilst B cell-specific IL-4Rα$^{-/-}$ mice did not produce IgG1 there was no significant decrease in parasite antigen specific splenocyte IL-10 production (although levels were rather low). Crucially, disease progression is not affected in these animals when compared with wild type mice and the animals went on to develop non-healing lesions following infection, even in the absence of parasite-specific IgG1. Therefore, considering that Alexander *et al* (2002) have demonstrated that global depletion of IL-4Rα in BALB/c mice confers full resistance, and that this

has been previously attributed to IgG1 production (Chu *et al,* 2010), the findings in the present study suggest that development of chronic infection during *L. mexicana* may not be simply be ascribed to IgG1, and that other factors must be also be involved. Indeed T cells, though not macrophages or neutrophils, responding to IL-4 have been shown to play a significant role in the non-healing response to *L. mexicana* (Bryson et al 2011)

As highlighted in Chapter 1, a number of studies have highlighted a protective role for IL-4 during *Leishmania* infection during the early stages of infection (Beidermann *et al*, 2001; Ehrchen *et al,* 2010). Biedermann *et al* (2001) first proposed that during *L. major* infection, early administration (8 h) of high doses of IL-4 could enhance DC production of IL-12 and would therefore promote Th1 cell immunity. Following this, it was then demonstrated *in vitro* by Yoa *et al* (2005) that IL-4 could instruct LPS stimulated DCs to suppress IL-10 production, upregulate IL-12 production and thus influence T helper cell differentiation. These studies were further supported by Ehrchen *et al* (2010), who linked the enhanced protective IL-12 production phenotype observed in resistant C57BL/6 mice with a "protective" peak of keratinocyte IL-4 expression during the first 24h of *L. major* infection, and associated this early IL-4 production with a healing response.

Taken together, these studies provide evidence to suggest that IL-4 responsive DCs are important for the generation of a Th1 healing response during *Leishmania* infection. Thus, the creation of DC specific IL-4R$\alpha^{-/-}$ BALB/c (Hurdayal *et al*, 2013) provides a useful tool to determine how IL-4/IL-13 can affect DC cytokine production and disease progression during infection. Hurdayal *et al* (2013) use these mice to demonstrate that mice lacking responsiveness to IL-4/IL-13 will be rendered

hyper-susceptible to *L. major* infection. DC specific IL-4Rα$^{-/-}$ developed larger lesions that contained more parasites than their littermate controls. Interestingly, these mice also produce less IL-12 and enhanced levels of IL-4, suggesting that IL-4/IL-13 does have an impact on Th1 associate cytokine and thus control of the disease.

The results of this preliminary study suggest that the effect of DC specific IL-4Rα$^{-/-}$ depletion has differing effects between *L. major* and *L. mexicana* infected mice. It is important to take into consideration that, because of rapid lesion growth (possibly due to concomitant infection), the time course of this study was greatly reduced and thus the results of this study must be interpreted with caution. However, these preliminary results suggest that depletion of IL-4Rα on DCs does not have a significant influence on early lesion growth or parasite burden when compared to littermate control mice. Splenocyte parasite specific cytokine responses were also unaltered which hints that IL-4 may not have such a profound impact on DC function following *L. mexicana* infection, than was observed during *L. major* infection (Hurdayal *et al,* 2013) where DC specific IL-4Rα$^{-/-}$ depleted mice were found to be hypersusceptible to infection.

In **Chapter 3** it was demonstrated that DC migration to the draining lymph node is impaired during *L. mexicana* infection. The present study demonstrates that the migration of IL-4Rα$^{-/-}$ DCs to the LN was reduced, regardless of infection or LPS-induced inflammation. Recently, Thomas *et al* (2012) have used a transcriptomics approach to study chemokine receptor expression by alternatively activated macrophages from WT and IL-4Rα-deficient animals. This study suggests that

expression of various chemokine and chemokine receptors is modulated upon IL-4 stimulation and that alternatively activated macrophages are unable to respond to numerous chemokines and do not migrate to LNs. Therefore, it could be possible that similar chemokine receptor regulation also occurs in IL-4Rα$^{-/-}$ DCs? Thus, the observed failure of DC migration may be attributed to down regulation of chemokine receptor expression, and if this were the case, inhibition of CCR7 expression would directly influence DC migration.

As has been previously discussed, Biedermann *et al* (2001) suggest that IL-4 is required to stimulate DC production of IL-12. With this in mind, a study of DC migration to the dLN and subsequent T cell activation during *Mycobacteria* infection (Khader *et al,* 2006), demonstrates that IL-12 is essential to stimulate DC migration. Therefore, it could possibly be the case that IL-12 has a similar role in *L. mexicana* infection and that inhibition of IL-4 stimulation in DCs may indirectly affect the migratory capacity of the cell? Further to this, if this coincides with already compromised DC migration, this could potentiate the effect and reduce the development of Th1 immunity even further. Therefore, further characterisation of DC kinetics throughout infection will be advantageous in understanding how DC motility is affected by the loss of IL-4Rα, and what this will mean for antigen presentation and T cell immunity.

Together, the results presented in this chapter suggest that mice with B cells that are non-responsive to IL-4 fail to produce IgG1, yet IL-10 production is not significantly compromised and disease pathology is not reduced. Additionally, depletion of IL-4Rα specifically on DCs suggests that IL-4 signaling, whilst important in driving DC

migration during inflammation, does not appear to be influential during *L. mexicana* infection.

Chapter 8. General Discussion

Honed over millennia, *Leishmania spp.* utilise an array of virulence mechanisms to evade host immunity, as discussed in Chapter 1. These mechanisms include modulating immune cell signaling (Cameron *et al,* 2005), expression of cell surface receptors/ligands, or cytokines (Contreras *et al,* 2014), and altering cell motility (Ato *et al,* 2006; Petritus *et al,* 2012). Each of these factors can impact upon host immunity and therefore determine parasite survival and disease progression. However, the mechanisms underlying these observations are ill-defined and questions about how *Leishmania mexicana* is able to evade host immunity to develop chronic disease have been an important area of research. Specifically, does the parasite modulate migration of immune cells, such as antigen presenting DCs, in a bid to circumvent the development of protective immunity? If so, how does *L. mexicana* mediate this evasion strategy and may cysteine protease B have a role in this process? Thus, if *L. mexicana* is able to alter cell migration, can we identify potential targets for new drugs to overcome this suppression of the host response? Additionally, might neutrophils and NETs aid in the development of chronic infections with *L. mexicana*? Finally, what role do cytokines, like IL-4 have in the progression of infection? Therefore, the main aim of this thesis was to determine whether *L. mexicana* might influence immune cell migration and to explore the mechanisms underpinning this.

As has been discussed in earlier Chapters, functional DC migration, antigen processing and presentation are essential for generating parasite-specific effector T cells. Some *Leishmania* species have been described as influencing DC recruitment into the infection site and their subsequent migration to lymphoid organs, but can *L. mexicana* affect the ability of DCs to activate T cells by modulating cell migration?

The results presented in Chapter 3 demonstrate that *L. mexicana* abrogates DC responsiveness to CCL19, reducing CCR7 expression and DC migration towards lymphatic vessels *in vivo* and subsequently reducing accumulation of DCs in the lymph node. This important observation suggests that, through preventing DC migration, *L. mexicana* is able to evade early recognition by the adaptive immune response, allowing early parasite development and development of chronic disease.

Such a finding provides the potential for therapeutic intervention. It was hypothesised that if DC migration to the LN was stimulated soon after infection this may be sufficient to overcome parasite-mediated evasion of immunity. The findings presented in Chapter 4 demonstrate that, whilst Th1 immune responses are moderately increased by treatment with R-848 – an inducer of DC activation and CCR7 expression – this fails to retard disease progression in susceptible BALB/c mice. These results suggested that the lack of CCR7 expression on parasite-containing DCs was not the only mechanism underlying the failure of migration and so further work explored the parasite-derived molecules that may alter DC behavior.

To explore this idea further, I next investigated how one *L. mexicana* virulence factor CPB, may affect CCR7 expression and DC migration (Chapter 5). Whilst CPB has been shown to modulate intracellular trafficking of a number of other surface receptors on antigen-presenting cells (Cameron *et al*, 2004; De Souza Leao *et al*, 1995), it was discovered that CPB does not change CCR7 expression on DCs exposed to parasites or to CPB itself. However, extracellular CPB appears to cleave CCL19 directly, removing one of the key pathways in driving DC migration.

Whilst direct cleavage of CCL19 may represent one mechanism through which parasites evade host immunity, DCs interacting with parasites early during infection do so in the context of other recruited immune cells, which may also influence DC function. Neutrophils are important in shaping the immune response (McFarlane *et al,* 2008; Tachini- Cottier *et al,* 2002; Charmoy *et al,* 2010) and Chapter 6 suggests that neutrophils may not only influence DC migratory behavior but also that NETs appear to facilitate transfer of parasite antigen to phagocytes, contributing to protective immunity.

Cytokines also play a key role in generation of protective immunity, and yet defining the role of certain cytokines, such as IL-4, and particularly their cellular targets in the early stages of the response has proven illusive. In Chapter 7, I demonstrated that the absence of IL-4R□ on DCs renders mice fully susceptible to *L. mexicana* infection, suggesting that IL-4/IL-13 may be important for stimulation of DC motility. Related studies also determined that mice in which B cells were unresponsive to IL-4 showed susceptibility to *L. mexicana* despite a lack of IgG1, suggesting that this antibody is not essential as suggested by others for pathogenicity in New World cutaneous leishmaniasis.

Therefore, the main finding of this work is that *in vitro* and *in vivo* DC migration is compromised by *L. mexicana* infection; an effect mediated (at least part) through the proteolytic activity of CPB on CCL19. Such a strategy may provide the parasite with an early advantage in establishing infection, and likely having a critical effect on the rapid development of an adaptive immune response.

The evidence provided here further demonstrates the importance of DC migration in generating protective immunity and suggests that a CPB-mediated degradation of CCL19 contributes to the lack of DC migration observed during *L. mexicana* infection. In addition, it should be considered that because CPB can be secreted and show activity beyond the infected host cell (Ilg *et al,* 1994), the protease could affect a whole host of chemokines and/or receptors, and inhibit the migration of non-infected, bystander immune cells. Indeed *in silico* analysis based on Cathepsin-L activity suggests that, as with CCL19, CPB has the potential to cleave CCL21, as shown in Figure 8.1 below.

Results:

>gi_6094386_sp_O00585_1_CCL21_HUMAN_RecName__Full_C_C_motif_chemokine_21__AltName__Full_6Ckine__AltName__Full_Beta_chemokine_exodı

rank	position	site	N fragment	C fragment	frequency score	similarity maxscore	similarity maxsite	average score	specificity
1	14 to 19	LAFG.IP	1.7 kD	12.9 kD	11.574	27.273	LFYEAP	315.657	>99%
2	66 to 71	PRKR.SQ	7.5 kD	7.1 kD	4.409	54.839	PKKEST	241.793	>95%
3	123 to 128	KRTE.RS	13.8 kD	0.9 kD	5.401	37.037	KAARKS	200.046	>95%
4	3 to 8	QSLA.LS	0.6 kD	14.0 kD	3.601	51.852	RGLSLS	186.709	>95%

Figure 8.1. Excerpt from full SitePrediction analysis of putative cleavage sites of CCL21 (mouse) (UniProt) by Cathepsin L (Human).

Scoring was carried out using the BLOSUM 62 matrix. Black boxes highlight putative cleavage positions and specificity score.

Other organisms such as Group A *Staphylococcus* (GAS) and *S. aureus* have been shown to produce enzymes capable of degrading chemokines during infection. GAS has been shown to produce scpC which can degrade CXCL1 and CCL2 (Hidalgo-Grass *et al,* 2006) and *S. aureus* can produce Staphopain A to cleave CXCR2 (Laarman *et al,* 2012). Through these mechanisms the bacteria can prevent neutrophil activation and recruitment, and subsequently can evade the innate immune

response. Smith *et al* (2005), have demonstrated that *Schistosoma mansoni* eggs can produce smCKBP, a chemokine binding protein, which can bind to CXCL8, CCL3 and CX3CL1 and can, in this fashion block their activity *in vivo*. Similarly, a number of viruses (Johnston *et al,* 2000) and the hookworm *Necator americanus* is capable of producing metalloproteases that cleave and inactivate a number of chemokines (Culley *et al,* 2000).

The failure of DC migration in early *L. mexicana* infection may account for the chronicity often associated with this infection and therefore represents a potential target for therapeutic targeting. Having shown that the inhibition of DC migration by *L. mexicana* was not solely due to reduced CCR7 surface expression, and that the parasite could also degrade CCL19, it is perhaps not surprising that R-848 showed only limited effects on protective immunity. However, a number of clinical studies have shown that subcutaneous injection of R-848 versus topical administration of Aldara (imiquimod) will have different immunological outcomes (Fehres *et al,* 2014), and therefore there is a possibility that topical administration of R-848 could have a different effect during *L. mexicana* infection.

An alternative therapeutic approach may be to block proteolytic activity at the site of infection to restore chemokine activity and DC migration. Breast cancer metastasis has been associated with a cathepsin-D-dependent degradation of CCL21 (Wolf *et al,* 2003), abrogating CCR7-binding, and this can be blocked therapeutically using pepstatin A. Crucially, a natural inhibitor of *L. mexicana* cysteine proteases has been identified (Denise *et al,* 2003) and infection of mice with parasites engineered to over-express this inhibitor generates a protective Th1 response (Bryson *et al,* 2009). Whether such protection is associated with enhanced DC migration remains to be

explored, but these findings suggest that targeting of chemokine-proteolytic activity represents an important area for therapeutic development in the future (Proudfoot *et al,* 2015)

Conversely, understanding the mechanism of immune-evasion associated with chronic *Leishmania* infection provides alternative routes to new medicines for other immune conditions such as arthritis and asthma. For example, the family of evasins, derived from the tick (*Rhipicephalus sanguineus*) bind and inhibit a range of chemokines and have been applied to a number of disease models (Deruaz *et al* 2008; Bonvin *et al*, 2014). Furthering our understanding of the underlying mechanisms of chemokine targeting by *L. mexicana* could provide similar approaches in controlling other diseases.

The aim of this project was to investigate whether *L. mexicana* could influence immune cell migration and identify the mechanisms underlying this. The results presented here demonstrate that DC migration following *L. mexicana* infection is modulated by several factors, especially through cysteine protease B degradation of CCL19, but also by interactions with neutrophils and their NETs and the actions of early IL-4α signaling. Thus, these findings may have wider implications in the way we think about the development of chronic disease, how parasite-derived products can modulate host immunity and how these factors can direct the development of new therapies for intracellular parasite infection.

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