Immune responses during cutaneous and visceral leishmaniasis

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SUMMARY

Leishmania are protozoan parasites spread by a sandfly insect vector and causing a spectrum of diseases collectively known as leishmaniasis. The disease is a significant health problem in many parts of the world, resulting in an estimated 1·3 million new cases and 30000 deaths annually. Current treatment is based on chemotherapy, which is difficult to administer, expensive and becoming ineffective in several endemic regions. To date there is no vaccine against leishmaniasis, although extensive evidence from studies in animal models indicates that solid protection can be achieved upon immunization. This review focuses on immune responses to *Leishmania* in both cutaneous and visceral forms of the disease, pointing to the complexity of the immune response and to a range of evasive mechanisms utilized by the parasite to bypass those responses. The amalgam of innate and acquired immunity combined with the paucity of data on the human immune response is one of the major problems currently hampering vaccine development and implementation.

INTRODUCTION

Leishmania are protozoan parasites shuttling between the sandfly vector where they multiply as free promastigotes in the gut lumen, and the mammalian host where they proliferate as obligatory intracellular amastigotes in the mononuclear phagocytes (Handman, 1999). Leishmaniasis is prevalent in Africa, Latin America, Asia, the Mediterranean basin and the Middle East. The cutaneous form (CL) of the disease accounts for more than 50% of new cases of leishmaniasis. The majority of cases of CL (90%) occur in Afghanistan, the Middle East and South America. Related diseases include diffuse cutaneous leishmaniasis (DCL) that arises in anergic hosts, and mucocutaneous leishmaniasis (ML) characterized by the late development of metastatic lesions and destruction of the mucous membranes. Visceral leishmaniasis (VL), also known as kala-azar, is the most severe and often fatal syndrome. The vast majority of all VL cases occur in five countries: India, Bangladesh, Ethiopia, Sudan and Brazil (Modabber, 2010). The disease is mostly zoonotic in origin and canine species are the main animal reservoir; however, humans are the only known hosts for *Leishmania donovani*. Between 20 and 60% (depending on geographical location) of VL patients infected with *L. donovani* develop a syndrome known as post kala-azar dermal leishmaniasis (PKDL), which appears within a few years of the complete cure of VL. PKDL patients are considered a major source of parasites for new infections due to the large number of parasites present in the skin.

Leishmaniasis has been classified as one of the most neglected diseases, and the estimated disease burden places it second in mortality and fourth in morbidity among the tropical infections (Bern *et al*. 2008). Leishmaniasis has also been identified as an emerging infection among travellers, and its treatment poses a challenge due to the unfamiliarity of physicians in non-endemic countries with symptoms, diagnosis and treatment options (Pavli and Maltezou, 2010). In general, leishmaniasis is a disease linked to poverty, and is associated with malnutrition, poor housing, illiteracy and lack of resources. The majority of the afflicted population cannot afford the cost of treatment, which surpasses a substantial percentage of household income and represents a significant financial burden. Hence, market forces do not drive the development of new treatments, either vaccines or drugs, as the poor return of funds invested in research and development remain unattractive to industry. Current chemotherapy for leishmaniasis includes pentavalent antimonials such as sodium stibogluconate and meglumine antimonite. This class of drugs has been recommended for the treatment of leishmaniasis for over 70 years. Second-line drugs used in the treatment of leishmaniasis include aromatic diamidines (pentamidine) and amphotericin B, but these drugs can be toxic and are characterized by severe side effects (Kedzierski *et al*. 2009).

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The development of a vaccine to prevent leishmaniasis has been a goal for almost a century, but currently no such vaccine exists. *Leishmania* vaccine development has proven to be a difficult and challenging task, which is hampered by inadequate knowledge of parasite pathogenesis and the complexity of immune responses needed for protection. These aspects are of key importance in the vaccine development process (Kedzierski *et al*. 2006).

The control of *Leishmania* infection in the mammalian host is mediated by cellular immune responses leading to macrophage activation and parasite killing. Although a strong humoral response is present during the infection, antibodies play no role in protection and are associated with the non-healing disease. Antileishmanial immunity is mediated via both innate (macrophages, dendritic cells (DCs) and neutrophils) and adaptive (T cells) immunity, but the $CD4^+$ T cell subset is crucial for resistance. In addition, cytokines participate in a network of interactions that induce and control the immune responses. Experimental studies using a leishmaniasis mouse model of disease gave rise to the T helper 1 (Th1)/Th2 paradigm of resistance and susceptibility associated with intracellular infection. This clear-cut dichotomy is mostly associated with CL, but is not so well defined for VL. Nevertheless, it is accepted that the nature of the T cell response is a crucial factor in resistance to the disease, despite evident differences in the responses observed between mouse experimental infection and human leishmaniasis.

CUTANEOUS LEISHMANIASIS

Innate immune responses

Macrophages play a pivotal role in *Leishmania* infection. Effective clearance of all forms of leishmaniasis depends on efficient elimination of parasites by activated macrophages. Macrophages are professional phagocytes and *Leishmania* utilizes their phagocytic function as a strategy for internalization and subsequent replication within the macrophage phagolysosomes (Reiner and Locksley, 1995). Thus, macrophages act as both host cells for leishmanial replication and effector cells that kill the parasites. Internalization of *Leishmania* by macrophages triggers the production of reactive oxygen species (Basu and Ray, 2005) and leads to the generation of nitric oxide (NO) (Liew *et al*. 1990) and *N*-hydroxy-L-arginine (LOHA) (Iniesta *et al*. 2001), which can mediate parasite killing. There are different requirements for effective killing of the different *Leishmania* species that cause CL. While NO and LOHA are sufficient for elimination of *Leishmania major* (Wei *et al*. 1995), a successful anti-*Leishmania amazonensis* response also requires superoxide production (Mukbel *et al*. 2007). Additionally, infection of macrophages leads to the production of pro-inflammatory cytokines that are implicated in parasite killing. Interleukin (IL)-12 is necessary for the leishmanicidal activity of macrophages, as it leads to up-regulation of interferon gamma (IFN-γ) by T cells and NK cells, generation of Th1-type responses and T cell-dependent and -independent macrophage activation leading to an increase of inducible nitric oxide synthase (iNOS) and NO production, and subsequent parasite elimination (Trinchieri, 1998). A subversive activity of *Leishmania* parasites in this process is the inhibition of IL-12 production, which down-regulates the immune response to infection (Ricardo-Carter *et al*. 2013).

Production of pro-inflammatory cytokines by macrophages results in the recruitment of pro-inflammatory cells such as neutrophils, mast cells and eosinophils to the site of infection.

Neutrophils are among the first cells recruited to the site of infection and are thought to participate in the containment of *Leishmania* parasites within an hour of infection (Belkaid *et al*. 2000). Neutrophils are a major component of innate immunity that protects against microbial infections (Segal, 2005). Published data on the involvement of neutrophils in *Leishmania* infection are contradictory, indicating either their role in resistance to leishmaniasis or disease-exacerbating activities (Peters and Sacks, 2009). However, it has been shown that in the context of infection initiated by the bite of an infected sandfly, neutrophils are recruited to the site of infection and phagocytose parasites, a process that is vital for disease progression (Peters *et al*. 2008). These findings suggest that neutrophils, apoptotic neutrophils in particular, are more likely to play a role in promoting disease progress, rather than resistance. However, other studies have found that neutrophils contribute to parasite killing through the release of neutrophil extracellular traps (NETs) (Guimaraes-Costa *et al*. 2009). Subsequent production of mast cell-derived mediators, immunoglobulin G-mediated mechanisms and cytokines/chemokines released by macrophages and neutrophils result in the recruitment of DCs, an important component linking the innate with the adaptive immune response against *Leishmania* (Reiner and Locksley, 1995).

The main function of DCs is the recognition and processing of foreign antigens, and subsequent presentation of antigen to T cells (Banchereau and Steinman, 1998), and as such they are considered to be gatekeepers in the defence against invading pathogens. Upon encountering antigen in the presence of pro-inflammatory cytokines, immature DCs residing in the skin undergo maturation and migrate to draining lymph nodes (Randolph, 2001). Skin DCs, Langerhans cells and dermal DCs are very efficient at presenting antigen to naïve T cells (Von Stebut, 2007). In the case of *Leishmania* infection, dermal DCs appear to present antigen directly to T cells (Ritter *et al*. 2004). Small numbers of parasites

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are taken up directly by dermal DCs shortly after infection (Ng *et al*. 2008), but the majority of the DCs become infected through contact with parasitized neutrophils (Peters and Sacks, 2009). Several weeks post-infection the number of DCs ($CD11c^+$ cells) in the lesion increases through recruitment (Belkaid *et al*. 2000) and infected DCs are able to prime naïve $CD4^+$ and $CD8^+$ T cells (Woelbing *et al*. 2006). Activated DCs migrate to draining lymph nodes where apart from T cells, they also activate resting NK cells and trigger IFN-γ production (Bajenoff *et al*. 2006). However, *Leishmania* parasites have evolved complex mechanisms to avoid DC functions, leading to the down-regulation of DC activation during infection. Amastigote infection of DCs results in reduced phosphorylation and degradation of vital molecules in Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT), nuclear factor (NF)-κB and Interferon Regulatory Factor (IRF) pathways (Soong, 2008), which leads to inadequate DC activation, T cell priming, impaired NK cell activation and suppression of IL-12 and IFN-γ production. These might be *Leishmania*-related general phenomena; however, there are species- and stage-specific differences in modulation of DC functions. While infection with *L. major* or *L. donovani* promastigotes led to production of IL-12 by murine DCs (Gorak *et al*. 1998; von Stebut *et al*. 1998), infection with *Leishmania mexicana* amastigotes did not lead to DC activation or IL-12 and other pro-inflammatory cytokines production (Bennett *et al*. 2001). Similarly, infection with *L. amazonensis* amastigotes leads to down-regulation of signalling events and impaired DC function (Xin *et al*. 2008). In humans *L. amazonensis* has been shown to use Langerhans cells to skew $CD4^+$ T cell function towards regulatory T (T reg) cells and to suppress protective responses (Silveira *et al*. 2008).

Amastigote uptake by DCs at the site of infection results in the up-regulation of IL-12 (Gorak *et al*. 1998), which is essential for parasite elimination within DCs (von Stebut *et al*. 1998) and for the effector functions of macrophages (Belkaid *et al*. 1998). Uptake of amastigotes by DCs also leads to surface up-regulation of MHC I, MHC II and co-stimulatory molecules. The ability of DCs to present antigens through the MHC I and II pathways leads to stimulation of *Leishmania*-specific $CD4^+$ and $CD8^+$ T cell responses (Flohe *et al*. 1997; Belkaid *et al*. 1998; von Stebut *et al*. 1998). Although other cell sub-sets, including macrophages and B cells, are able to present leishmanial antigens, antigen presentation by DCs is essential for acquired resistance to *Leishmania*.

Adaptive immune response

T cells play an essential role in the generation of effector and memory responses to intracellular pathogens. In general terms, protective immunity is associated with a cell-mediated immune response, whereas non-protective responses have a strong humoral component in the absence of cell-mediated immunity. The protection against CL is intimately linked to development of Th1-type immunity and IFN-γ production. Experimental studies established a clear-cut dichotomy between Th1-mediated protection and Th2-mediated susceptibility. In resistant C57BL/6 mice, resolution of the disease is mediated as a consequence of IFN-γ release by Th1 cells and up-regulation of NO in macrophages that harbour parasites (Bogdan *et al*. 2000). Conversely, persistence of lesions in susceptible BALB/c mice is due to $CD4^+$ T cell differentiation to Th2-type effector cells and the production of IL-4, which in turn promotes antibody responses and suppresses macrophage activation, resulting in parasite survival and replication (Scott, 1989). The Th1 response is linked to IFN-γ production; however, it is functionally heterogeneous. It has been shown that a high frequency of $CD4^+$ T cells producing only IFN-γ is not sufficient for resistance to infection. The quality and magnitude of the response are crucial factors influencing a protective outcome and are controlled by the type of antigen-presenting cells (APCs), amount of antigen and the duration for which it is presented to the immune system as well as cytokine milieu (Iezzi *et al*. 1998; O'Garra, 1998; Steinman and Hemmi, 2006). The Th1 response mounted by $CD4^+$ T cells which are single-positive, i.e. producing only IFN-γ or tumour necrosis factor (TNF), have limited aptitude to develop into memory cells compared with IL-2 producing cells. Hence, their capacity to provide long-term durable protection is rather limited. On the other hand, IFN-γ and TNF are known to synergize in order to more efficiently kill parasites (Bogdan *et al*. 1990); therefore, multifunctional $CD4^+$ T cells that simultaneously produce multiple cytokines are more likely to be involved in resistance to infection. Indeed, the frequency of multifunctional $CD4^+$ T cells ($IFN\text{-}\gamma^+$ TNF^+ $IL\text{-}2^+$) correlates with the degree of protection following vaccination (Darrah *et al*. 2007). These data indicate that functional heterogeneity of the Th1 response to *Leishmania* plays a significant role in resistance to infection.

The Th1/Th2 dichotomy has been questioned in recent times since there is accumulating evidence that early IL-4 responses might not be required to promote susceptibility and there is considerable complexity in the mechanisms responsible for acquired immunity (Sacks and Noben-Trauth, 2002). Resistant C57BL/6 mice produce IL-4 early at the onset of the infection. This increase in IL-4 did not impact the mounting of an unimpaired Th1 response and disease resolution (Scott *et al*. 1996). In several cases, resistance to infection in BALB/c mice following immunization has not been linked to a strong Th1 response (Uzonna *et al*. 2004*b*;

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Kedzierski *et al*. 2008), and in some cases high pre-challenge IFN-γ levels did not correlate with protection (Stober *et al*. 2005). A recent report implicated keratinocytes and epidermal cytokine expression as decisive factors in the generation of Th1 immunity (Ehrchen *et al*. 2010). The critical events that influence Th1/Th2 differentiation were thought to occur in the lymph nodes early during infection; however, it was also acknowledged that the skin, as a primary site of infection, could influence the immune response (Sunderkotter *et al*. 1993). During the first few hours of infection, *Leishmania* induces several cytokines in keratinocytes and the gene expression profile of cells differs in susceptible and resistant mice. In particular, production of IL-4 by epidermal cells can explain the somewhat controversial role this cytokine plays in induction of Th1/Th2 responses. While IL-4 is associated with a Th2 response and susceptibility to leishmaniasis (Himmelrich *et al*. 2000), it is also able to induce the production of IL-12 by DCs, but only when present early during the infection (Biedermann *et al*. 2001). Therefore, an early transient IL-4 production by keratinocytes is essential for induction of Th1 response against *L. major*, by acting in a paracrine fashion on DCs, which then produce IL-12 upon migration to the lymph node (Ehrchen *et al*. 2010). It has also been shown that IL-6, a major inflammatory cytokine, plays an important role in *Leishmania* protection. High levels of IL-6 of keratinocyte origin have been detected in resistant strains, and mice with IL-6 deficiency in the non-haematopoietic compartment display a Th2 skewing and non-healing phenotype (Ehrchen *et al*. 2010).

Susceptibility and resistance to infection are also influenced by T reg cells ($CD4^+CD25^+$), which reside in the skin where they suppress harmful immune responses to infectious agents, counteract inflammatory responses and limit tissue damage (Belkaid and Rouse, 2005). During *L. major* infection, T reg cells accumulate in the dermis where they suppress the ability of the effector T cells to eliminate parasites. This process has been linked to the production of IL-10 (Belkaid *et al*. 2002*a*), a cytokine that is also implicated in the maintenance of parasite persistence (Belkaid *et al*. 2001). High levels of IL-10 produced by antigen-driven T reg cells lead to lack of vaccine efficacy despite the presence of strong Th1 responses (Stober *et al*. 2005). In humans, T reg cells have been found in lesions of CL patients (Campanelli *et al*. 2006) and have been implicated in immunopathogenesis of the cutaneous infection (Bourreau *et al*. 2009). It has been demonstrated that $CD4^+CD25^+Foxp3^+$ T reg cells are involved in a rapid loss of resistance to infection in immune animals following inoculation with a killed parasite vaccine (Okwor *et al*. 2009). These data clearly point to the important regulatory role that T reg cells play in resistance and susceptibility to CL.

Cytotoxic activity and cytokine production are two major effector functions of $CD8^+$ T cells that contribute to the disease outcome in *Leishmania* infections. The majority of data do not indicate a protective role for $CD8^+$ T cells in controlling primary infection (Huber *et al*. 1998). However, they clearly play a role in resistance to infection by inducing Th1 responses via cytokine production (IFN-γ) or in recall responses to secondary infection (Muller *et al*. 1993). IFN-γ-producing $CD8^+$ T cells are fundamental for the development of a Th1 response and thus contribute to healing in C57BL/6 mice (Belkaid *et al*. 2002*b*; Uzonna *et al*. 2004*a*). Besides cytokine production, $CD8^+$ T cells are thought to participate in controlling the infection through cytotoxic mechanisms, such as granzyme and perforin production and Fas/FasL pathways (Ruiz and Becker, 2007). In human CL, recruitment of $CD8^+$ T cells producing granzyme A to the site of infection is associated with tissue damage, despite this being a consequence of anti-parasitic action (Faria *et al*. 2009). Also in *Leishmania braziliensis*-caused CL, $CD8^+$ T cells were shown to play a harmful role contributing to disease immunopathology via their cytotoxic activity leading to tissue destruction (da Silva Santos *et al*. 2013). It is still not known what is the exact route of $CD8^+$ T cell activation in leishmaniasis, since the parasites reside in a parasitophorous vacuole inside the host macrophages and it is not clear how these cells present antigen through MHC I (Bertholet *et al*. 2006). The most likely mechanism is cross-presentation, which has been well documented for macrophages and DCs (Rodriguez *et al*. 1999; Houde *et al*. 2003), a process suggested to occur during *Leishmania* infection (Bertholet *et al*. 2006) and one which the parasite is also able to block in order to evade immunity (Matheoud *et al*. 2013).

Development of humoral immune responses is often linked to susceptibility to *Leishmania* infection, and in general antibodies are not considered to be a major factor in resistance to disease. B cell depletion using anti-IgM antibodies enhanced resistance to *Leishmania* in BALB/c mice (Sacks *et al*. 1984). Administration of IL-7, a B cell stimulant, to BALB/c mice increased B cell numbers and enhanced disease severity (Hoerauf *et al*. 1995). Furthermore, B cell-deficient (μMT) mice lacking B cells through the targeted disruption of the immunoglobulin M locus are more resistant to infection with *Leishmania* than their wild-type counterparts (Smelt *et al*. 2000). In addition, adoptive transfer of B cells and serum into BALB/c μMT mice has shown that antigen presentation of specific B cells rather than immunoglobulin effector functions are involved in the susceptible phenotype of BALB/c (Ronet *et al*. 2008). Conversely, μMT mice on both C57BL/6 and BALB/c genetic backgrounds showed no difference in disease phenotype and $CD4^+$ T cell polarity when compared with

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wild-type hosts (Brown and Reiner, 1999). Recent studies demonstrated that B cells are required for susceptibility and the development of Th2 cell responses in BALB/c mice during *L. major* infection (Ronet *et al*. 2008). The ability of B cells to skew the immune response towards a Th2 phenotype was linked to their capacity to present antigen to T cells. In addition, it has been shown that IL-10 produced by B cells may play a role in susceptibility to cutaneous infection by inhibiting IL-12 production by DCs *in vitro* (Ronet *et al*. 2010). Thus, the importance of B cell-mediated responses in *Leishmania* infection is controversial, but evidence points toward the involvement of B cells in disease susceptibility, at least in the mouse model of disease.

Cytokines

As described above, a wide range of cytokines and chemokines are involved in the immune response to *Leishmania* including, but not limited to, IL-4, IL-10, IL-12, IL-13, TNF and IFN-γ. The profile and timing of cytokine production correlates with the clinical outcome of *Leishmania* infection. A variety of immune cells express cytokines, mostly $CD4^+$ T cells (Th1 and Th2), but also $CD8^+$ T cells, $CD4^-CD8^-$ double-negative T cells (Gollob *et al*. 2008), NK cells, DCs and macrophages (Liese *et al*. 2008), mast cells (Maurer *et al*. 2006) and regulatory B cells (Ronet *et al*. 2010).

The exemplary Th2 cytokine in leishmaniasis is IL-4, which drives the Th2 response and promotes susceptibility through inhibition of macrophage activation and abrogation of IL-12 expression. The role of IL-4 in susceptibility to *Leishmania* has been illustrated in studies using transgenic and knockout mice. C57BL/6 IL-4 transgenic mice are more susceptible to infection than wild-type mice. Targeted disruption of the IL-4 gene or depletion of IL-4 in susceptible BALB/c mice renders them more resistant to *L. major* infection (Kopf *et al*. 1996). Additionally, disruption of the IL-4 receptor on $CD4^+$ T cells promotes resistance in BALB/c mice (Radwanska *et al*. 2007). However, the role of IL-4 as a major factor contributing to the disease susceptibility is controversial. One study indicated that BALB/c IL-4-deficient mice remained susceptible to disease in the absence of this cytokine (Noben-Trauth *et al*. 1996), whereas other studies showed that the same mice were resistant to *Leishmania* infection (Kopf *et al*. 1996; Alexander *et al*. 2002). These data question whether cytokines other than IL-4 might affect Th1 development during the infection. More recently, IL-4 has been identified as a negative regulator of chemokine production involved in Th1-type cell recruitment to the site of infection (Lazarski *et al*. 2013). Short-term blocking of IL-4 led to changes in Th1-associated chemokine gene expression and correlated with increased accumulation of IFN-γ producers. Therefore, elevated expression of IL-4 is thought to limit antimicrobial Th1 response in the inflamed tissue and prolong pathogen survival.

IL-13 shares a number of characteristics with IL-4 and both share a common signalling pathway through IL-4 receptor alpha (Brombacher, 2000). IL-13 has been demonstrated to have disease-promoting properties and to act independently of IL-4 (Matthews *et al*. 2000; Alexander *et al*. 2002), indicating that IL-13 and IL-4 effects might be additive. High levels of IL-13 might prevent the onset of Th1 response by inhibiting IL-12 production by macrophages and skew immune responses towards deleterious Th2-type profiles. In *L. mexicana*-induced disease, studies with IL-13 knockout mice implicated this cytokine in preventing disease resolution by inhibiting IL-12R expression (Alexander *et al*. 2002).

IL-10 is a major immunosuppressive cytokine in leishmaniasis, and as already discussed, is essential for parasite persistence (Belkaid *et al*. 2001) and can exacerbate infection (Murphy *et al*. 2001; Belkaid *et al*. 2002*a*). It is a potent suppressor of macrophage activation, inhibits DC maturation (O'Garra and Vieira, 2007) and is produced by a plethora of cells of the immune system (Moore *et al*. 2001). The ability of vaccinated mice to down-regulate IL-10 secretion has been linked to protection following inoculation with SIR-2-deficient *Leishmania infantum* parasites (Silvestre *et al*. 2007) and a phosphomannomutase (PMM) knockout line of *L. major* (Kedzierski *et al*. 2008). IL-10 knockout mice are highly resistant to *L. major*, whereas IL-10 transgenic mice on the resistant background become susceptible (Groux *et al*. 1999; Kane and Mosser, 2001). IL-10's crucial role in suppression of the immune response has been demonstrated in *L. mexicana* and *L. amazonensis* infections, although effective resolution of infection with these New World species requires neutralization of both IL-4 and IL-10 (Padigel *et al*. 2003). It has also been shown that IL-10 differentially influences the quality, magnitude and protective efficacy of Th1 cells depending on the vaccine platform (Darrah *et al*. 2010). Interestingly, co-expression of IL-10 and IFN-γ by Th1 $CD4^+$ cells prevents pathogen eradication and contributes to chronic infection (Anderson *et al*. 2007). IL-10 secreted by T cells has been shown to affect immune activation early in infection and a lack of T cell-specific IL-10 leads to enhanced protection following vaccination (Schwarz *et al*. 2013).

IL-9 also plays a role in *Leishmania* disease susceptibility. Produced mainly by Th2 clones (Demoulin and Renauld, 1998), induction of IL-9 can be either IL-4 dependent or independent (Kopf *et al*. 1993; Monteyne *et al*. 1997). During *L. major* infection, IL-9 synthesis was observed from 4 weeks onward only in susceptible BALB/c, but not in

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resistant C57BL/6 or DBA mice (Gessner *et al*. 1993*a*; Nashed *et al*. 2000). IL-9 neutralization in BALB/c mice resulted in a diminished Th2 response and a shift towards protective Th1 responses. This led to enhanced effector functions, including increased NO production by macrophages, implicating IL-9 as a susceptibility factor in leishmaniasis (Arendse *et al*. 2005).

Transforming growth factor-β (TGF-β) is a regulatory cytokine that controls initiation and resolution of inflammatory responses (Li *et al*. 2006). Different *Leishmania* species have been shown to induce TGF-β production from macrophages and release the active form of TGF-β from the latent complex (Mougneau *et al*. 2011). This cytokine is important for determining susceptibility to experimental leishmaniasis (Barral-Netto *et al*. 1992), and anti-TGF-β treatment promotes resolution of *L. major* infection in mice by augmenting NO production (Li *et al*. 1999). Overall, mouse studies have indicated that TGF-β inhibits Th1 responses and leads to increased susceptibility to leishmaniasis. This is achieved by suppression of NO production and inhibition of TNF and IFN-γ.

Recently, increased levels of IL-17 have been detected in patients with CL (Bacellar *et al*. 2009). The most prominent role of IL-17 is the induction of pro-inflammatory responses via production of cytokines such as IL-6, TGF-β or TNF. In the absence of IL-10, *L. major*-infected mice display increased levels of IL-17 and neutrophil infiltration. It has been postulated that IL-17 exacerbates pathology and its production is up-regulated by IFN-γ and controlled by IL-10 (Gonzalez-Lombana *et al*. 2013).

Cytokines with the ability to influence Th1 development, such as IL-12 or IFN-γ, play a protective role in leishmaniasis. IL-12's capacity to redirect early Th2 responses and promote resistance is well documented (Heinzel *et al*. 1993). IL-12 promotes resistance through macrophage activation and NO production, and is necessary for the priming of naïve T cells towards the Th1 pathway. Resistant mice depleted of IL-12 through the use of anti-IL-12 antibodies become more susceptible to infection, and administration of IL-12 to susceptible mice promotes resistance to infection (Heinzel *et al*. 1993). In addition, genetic disruption of the *Il12* gene leads to up-regulation of deleterious IL-4 responses and the establishment of progressive disease (Mattner *et al*. 1996). It has been suggested that IL-12 is required for optimal proliferation and production of IFN-γ by Th1 cells, both of which are significantly enhanced in the presence of IL-12, or that IL-12 can promote Th1 cell survival (Scott *et al*. 2004). Recent data indicate that the central memory $CD4^+$ T cells generated during *L. major* infection require IL-12 for IFN-γ production and differentiation into Th1-type, and in the absence of IL-12 these cells became IL-4 producers (Pakpour *et al*. 2008). The majority of IL-12 is produced by APCs such as macrophages, DCs and neutrophils (von Stebut and Udey, 2004); however, *L. major* has the ability to selectively block IL-12 production by macrophages (Carrera *et al*. 1996). DCs are the major source of IL-12 in leishmaniasis, which acts in combination with DC-derived IL-1α/β to influence Th1 development and promote resistance to cutaneous infection (Von Stebut *et al*. 2003).

Similarly to IL-12 deficiency, during IFN-γ deficiency the immune response will default to a Th2-type profile, leading to susceptibility to *L. major* (Wang *et al*. 1994). NK cells are the primary source of early IFN-γ production (Scharton and Scott, 1993), which plays a role in rapid development of Th1 response. Nevertheless, these cells are not essential for resistance to the cutaneous infection, since efficient IL-12-dependent IFN-γ production by $CD4^+$ T cells has been reported in the absence of NK cells (Satoskar *et al*. 1999). IFN-γ is a key cytokine that triggers the antileishmanial functions of macrophages via induction of NO production, and can activate macrophages alone or in synergy with TNF or IL-7 (Nacy *et al*. 1991; Gessner *et al*. 1993*b*). Resistant mice display elevated levels of IFN-γ compared with susceptible mice, while targeted disruption of the IFN-γ gene (Wang *et al*. 1994) or disruption of the ligand binding chain of the IFN-γ receptor (Swihart *et al*. 1995) in C57BL/6 mice results in increased susceptibility to *Leishmania* infection. However, contradictory data on the role of IFN-γ exist as some studies show that administration of IFN-γ to BALB/c mice at the time of infection does not affect the susceptibility of BALB/c mice to leishmaniasis (Sadick *et al*. 1990). Additionally, non-healing lesions in C57BL/6 mice were observed despite a strong Th1 response characterized by high IFN-γ, NO expression and low IL-4 production, but in the presence of higher IL-10 and Foxp3 mRNA expression (Anderson *et al*. 2005).

TNF is a pro-inflammatory cytokine produced primarily by activated macrophages, and is also produced by fibroblasts, T and B cells. It mediates resistance by controlling intracellular pathogen replication as well as limiting the duration of the inflammatory response (Havell, 1989). Synergizing with IFN-γ, TNF-α activates macrophages to exert iNOS-dependent leishmanicidal activity (Liew *et al*. 1990). Mice resistant to *Leishmania* produce high levels of TNF in the draining lymph nodes, whereas susceptible mice produce no or minimal TNF (Titus *et al*. 1989).

IL-27 is a cytokine produced upon exposure to inflammatory stimuli and is functionally and structurally related to IL-12 (Boulay *et al*. 2003). It has been implicated in regulation of T cell functions and IFN-γ production and, as a consequence,

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in promoting Th1 responses (Chen *et al*. 2000). Resistant mice lacking WSX-1 (a component of the IL-27 receptor) produce increased levels of IL-4 following *L. major* infection and a delayed Th1 response (Yoshida *et al*. 2001). However, the requirement for IL-27 appears to be transient and important only early in infection since WSX-1 knockout mice are able to control lesion development and resolve infection (Artis *et al*. 2004). IL-23 is a pro-inflammatory cytokine that also shows homology to IL-12 (Oppmann *et al*. 2000). IL-23-deficient mice showed increased susceptibility to bacterial and parasitic infections (Tan *et al*. 2009) and IL-23 is involved in regulation of IFN-γ production (Langrish *et al*. 2004). In leishmaniasis, IL-27 and IL-23 might play a complementary protective role with other Th1 cytokines. Human patients with *L. major* infection and healing CL lesions display elevated levels of IL-27 and IL-23 compared with patients with non-healing lesions (Tolouei *et al*. 2012).

Type I IFNs (IFN-α/β) are pro-inflammatory cytokines that are involved early in *L. major* infection as regulators of the innate response, NO production and IFN-γ expression (Diefenbach *et al*. 1998). Type I IFNs signal through STAT2, and during CL infection reduced STAT2 phosphorylation and increased degradation have been detected due to the activity of parasite proteases (Xin *et al*. 2008).

Taken together, the vast array of immune cells, chemokines (Oghumu *et al*. 2010) and cytokines involved in the immune response to *Leishmania* clearly highlights the complexity of the disease. The murine model of CL, which mimics many aspects of the human disease, has been used to dissect the role of cytokines and T helper cell responses. In human CL a clear dichotomy in T cell responses has not been reported, with patients displaying mixed Th1 and Th2 immune responses (Ajdary *et al*. 2000). Similarly, in human VL there is no strong association between Th1 responses and resistance to disease, with patients showing co-existing Th1- and Th2-type responses (Khalil *et al*. 2005). It appears that in humans, the outcome of CL disease is influenced by the balance between the two T cell populations and is further affected by the host genetic factors, inoculum size and parasite strain.

VISCERAL LEISHMANIASIS

Visceral leishmaniasis results from infection with the *Leishmania* species *L. donovani* and *Leishmania infantum* (*chagasi*). Parasites disseminate from the site of infection in the skin to reside and multiply within macrophages of the liver, spleen and bone marrow (Leclercq *et al*. 1996). The majority of people infected with visceralizing *Leishmania* species experience asymptomatic infection and only a small proportion of infections lead to clinically severe disease. However, when left untreated, clinical VL manifests as systemic chronic unresolving infection, which is usually fatal. Patients who recover from VL display immunity to re-infection, which suggests that the development of vaccines that provide clinical protection is a feasible goal. Immunocompromised individuals are susceptible to infection, and VL species are significant opportunistic pathogens during HIV infection (Ali, 2002). Together this indicates an important role for the host immune response during infection. The underlying factors that influence disease susceptibility are not entirely understood, but host genetic factors clearly play a role in determining the outcome of infection. The presence of the *Slc11a1* gene is associated with protection against *Leishmania* infection, as well as other intracellular pathogens (Vidal *et al*. 1995). This gene encodes a proton-coupled bivalent cation antiporter (Goswami *et al*. 2001) present in the phagosome membrane of macrophages (Searle *et al*. 1998). Slc11a1 is integral for regulating many cellular functions in macrophages, including cytokine production and antigen processing (Blackwell *et al*. 2001), and may also play a role in MHC II expression in DCs (Stober *et al*. 2007). Sca11a1 mutant mice are susceptible to *Leishmania* infection, and experimental VL infection of these mice leads to high parasite burdens in the visceral organs. Interestingly, parasite infection resolves in the liver in a manner determined by MHC haplotype (Leclercq *et al*. 1996), indicating a role for acquired immune responses in the control of parasite burden. In contrast to mice, polymorphisms in humans are confined to the promoter region of the *Sca11a* gene (Blackwell *et al*. 1995). Genetic linkage analysis has demonstrated an association between VL patients and polymorphisms in the 5′ (CA) repeat in the *Slc11a1* promoter (Goswami *et al*. 2001).

Experimental models of VL: insights into organ-specific immunity

Clinical studies examining the immune response to VL infection are limited by the difficulty in directly accessing infected tissues in patients. Many studies have investigated the systemic response to VL infection by examining circulating peripheral blood mononuclear cells (PBMCs) and serum cytokine levels. A key feature of VL patients is a depressed cell-mediated immune response, as PBMCs taken from VL patients do not proliferate *in vitro* and do not produce IFN-γ when stimulated with leishmanial antigens (Sacks *et al*. 1987). This has limited the utility of *in vitro* approaches; however, the recent development of whole-blood assays to detect cytokine production from infected patient samples could be applied to study immune correlates of disease status (Singh *et al*. 2012). To better understand the immune response to VL, experimental murine models of infection have been developed, as rodents

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are competent hosts for both *L. donovani* and *L. infantum*. These models have provided much insight into the organ-specific immune responses generated in the bone marrow, liver and spleen during VL. In addition, low-dose infection models using the infective metacyclic form of the parasite have been developed, which more accurately reflect the natural route of transmission (Ahmed *et al*. 2003).

Cytokine responses during VL

The majority of people infected with visceralizing *Leishmania* maintain an asymptomatic infection, but the mechanisms that mediate effective control of the disease are relatively unknown. A strong cytokine response is induced during VL and the production of IFN-γ appears crucial for the control of parasites and the development of resistance to infection (Squires *et al*. 1989). However, multiple cytokines and chemokines are produced in response to VL infection with elevated levels of IFN-γ, TNF, IL-6, IL-8, IL-10, IL-12, IL-15, IL-18, IL-33, IP-10 and MIG observed in the serum of VL patients (Sundar *et al*. 1997; Kurkjian *et al*. 2006). Thus, active infection is not due to an absence of a pro-inflammatory response, but is associated with the presence of both Th1 and Th2 cytokines. Thus the Th1/Th2 paradigm, which is thought to be important in CL, does not have such a clear role in determining the resistance/susceptibility profiles in human infection or in experimental models of VL (Miralles *et al*. 1994). Whilst clinical studies using samples from the peripheral blood of patients are informative, they may not necessarily reflect the events or immune mechanisms occurring in infected visceral organs. Studies with experimental rodent models demonstrate that organ-specific immune responses play a significant role in host defence of VL with defined patterns of tissue tropism and differential responses developing in the liver and spleen (Engwerda and Kaye, 2000).

Adaptive immune system: contributions of B and T cells to VL

B cells are not considered to play a significant protective role during *Leishmania* infection and have been implicated in exacerbating VL clinical disease (Galvao-Castro *et al*. 1984; Bohme *et al*. 1986). In contrast, T cells are critical for effective antileishmanial host responses. Immunocompromised mice lacking functional T cells, such as nude mice (Stern *et al*. 1988), severe combined immunodeficiency (SCID) mice (Kaye and Bancroft, 1992) and recombinase activating gene (RAG) knockout mice (Alexander *et al*. 2001) all show enhanced susceptibility to *L. donovani* infection, which can be overcome via reconstitution of T cell populations. Effector $CD4^+$ T cells are responsible for the production of cytokines that are critical for the activation of macrophages and the initiation of effective host protective responses. Cytotoxic $CD8^+$ T cells play a host protective role, and are required for effective clearance of parasites (Stern *et al*. 1988) and the generation of memory responses (Stager *et al*. 2000). Antigen-specific $CD4^+$ and $CD8^+$ cells are activated during infection, in both humans (Mary *et al*. 1999) and mice (Joshi *et al*. 2009), and are required for optimal host response to infection. Administration of antigen-specific $CD8^+$ T cells to *L. donovani*-infected mice significantly decreased parasite burdens in the liver and spleen (Polley *et al*. 2006), and the induction of $CD8^+$ T cell responses is being explored as a therapeutic intervention (Maroof *et al*. 2012). Interestingly, in an intradermal model of VL the clearance of parasites from the skin correlated with the infiltration and activation of both $CD4^+$ and $CD8^+$ T cells, analogous to the initiation of inflammatory responses and resolution observed in cutaneous infection (Ahmed *et al*. 2003).

VL and the liver: the granuloma response

The hallmark clinical manifestation of VL is a gross enlargement of the abdomen due to splenomegaly and hepatomegaly, and is observed in almost all VL patients. In experimental mouse models, hepatosplenomegaly is also a feature, and is associated with parasite infection of these tissues. Infection of the liver is evident at one week following *L. donovani* inoculation, peaking at 3–4 weeks post-infection and then resolving with minimal damage to the tissue (Polley *et al*. 2006). This acute resolving infection of the liver is associated with initial dominant reactive oxygen intermediate (ROI) and iNOS responses (Murray and Nathan, 1999). Liver parasite burdens are initially higher in mice deficient in phagocyte oxidase (gp91 $phox^{-/-}$) or nitric oxide synthase 2 ($NOS2^{-/-}$); however, gp91 $phox^{-/-}$ are able to resolve infection in the liver, whilst $NOS2^{-/-}$ sustain a progressive infection (Murray and Cartelli, 1983). This indicates that macrophages use both reactive oxygen and nitrogen intermediates in the initial effort to limit *L. donovani* replication in the liver, with RNIs appearing to play a more critical role in the resolution of liver parasite burdens.

Effective immune responses to VL in the liver are critically dependent on the formation of granuloma structures, which serve to coordinate and deliver cellular and soluble host defence factors to the infected tissue. The granuloma environment produces a focus for antileishmanial immune mechanisms, in terms of activating and sustaining appropriate parasite killing. During human VL, the presence of granulomas in the liver correlates with the ability to control and maintain infection

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at a sub-clinical level. In experimental models of VL, liver granulomas increase in number and size in the first month, leading to the clearance of parasites and the resolution of infection during the second month of infection (McElrath *et al*. 1988). Whilst the majority of parasites are cleared from the liver, sterile cure is never achieved, though the liver is resistant to re-infection. The induction of immunosuppression can re-activate infection, which has been observed in the case of HIV patients (Lachaud *et al*. 2009) or patients receiving immunosuppressive therapies following organ transplant (Antinori *et al*. 2008).

The core of the liver granuloma develops from tissue resident Kupffer cells that are recruited from the sinusoids during the acute phase of the inflammatory response (Beattie *et al*. 2010*a*). Kupffer cells are the major phagocytic population within the liver and the prime target for *Leishmania* infection. The generation of antileishmanial responses in the infected Kupffer cell is dependent on granuloma formation to provide the microenvironment for intracellular *L. donovani* killing (Murray, 2001). Infected Kupffer cells fuse with other mononuclear phagocytic cells to form the core of the granuloma, resulting in the secretion of chemokines and the infiltration and recruitment of leucocytes. Monocytes and neutrophils migrate to the liver within the first few days of infection and form a cellular mantle around the infected Kupffer cells in the developing granuloma. Experiments using depleting monoclonal antibodies towards monocytes and neutrophils demonstrate that these cells are essential for parasite killing as the maturation of hepatic granulomas is delayed in their absence (Cervia *et al*. 1993). The arrival of mononuclear cells leads to the recruitment of $CD4^+$ and $CD8^+$ T cells, which are essential for intact granuloma responses (Murray *et al*. 1987). B cells accumulate in granulomas over time in an antigen-independent manner and engage in long-lasting interactions with T cells (Moore *et al*. 2012). Interestingly, histological analysis of liver tissue shows that the formation and maturation of granulomas is asynchronous with mature granulomas possessing complete mononuclear cell cuffing observed alongside infected Kupffer cells that have failed to initiate granuloma formation. Mechanisms regarding the differential timing of granuloma formation and the inability of some infected Kupffer cells to induce appropriate host defence responses are not well understood. Upon resolution of infection, empty or sterile granulomas are evident in the mouse model, which then undergo an involution phase, restoring normal liver tissue function (Murray, 2001).

Chemokines and chemokine receptors have an important role in the development of protective immune responses in the liver due to their ability to attract Th1 cytokine-producing cells. Increased production of CCL3 (MIP1a), CCL2 (MCP-1) and CXCL10 (IP-10) occurs in the liver early during infection, and these factors are most likely produced by the infected Kupffer cell (Cotterell *et al*. 1999). The central role of chemokines in granuloma formation is highlighted by experiments demonstrating that administration of CCL2, CCL3 or IP-10 during experimental VL infection results in accelerated granuloma maturation in the liver and reduced parasite burdens (Dey *et al*. 2007). Furthermore, mice lacking CCL3 or its receptor CCR5 show enhanced susceptibility to *L. donovani* infection (Sato *et al*. 1999). Initial chemokine production and cell recruitment to the granuloma is T cell-independent, but sustained chemokine production and granuloma maturation requires the presence of infiltrating T cells.

Both $CD4^+$ and $CD8^+$ T cells are critical for granuloma formation, and the increase in $CD4^+$ and $CD8^+$ T cell numbers in the liver during VL infection may reflect expansion of resident populations as well as recruitment from the spleen (Stanley and Engwerda, 2007). During *L. donovani* infection T cells undergo high rates of apoptosis (Alexander *et al*. 2001), suggesting that immune responses are continually generated throughout the course of infection rather than being governed by long-lived effector T cell populations. In animals that lack T cells, such as SCID mice, the absence of sustained chemokine production results in a failure of granuloma formation and the uncontrolled growth of parasites in the liver (Engwerda *et al*. 1996). Nude mice, which lack functional T cells, also fail to control parasite growth and T cell transfer can restore resistance (Stern *et al*. 1988). $CD8^+$ T cells contribute to the control of liver parasite burdens through their role in granuloma formation (McElrath *et al*. 1988; Stern *et al*. 1988) and are essential for control in the liver during re-challenge experiments (Murray *et al*. 1992). The activity of $CD8^+$ T cells may involve perforin and FasL-dependent lysis of parasitized macrophages as well as the secretion of pro-inflammatory cytokines and chemokines (Tsagozis *et al*. 2003). The dynamics of $CD8^+$ effector T cells in the liver during *L. donovani* infection have been visualized using intra-vital two-photon microscopy and $CD8^+$ T cells were observed to accumulate in granulomas in an antigen-specific manner (Beattie *et al*. 2010*a*). This study also demonstrated that infected Kupffer cells are the main APC for $CD8^+$ T cells in the liver, and suggest that a sustained interaction with antigen-specific $CD8^+$ T cells may instigate lysis of the infected host cell (Beattie *et al*. 2010*a*). *However*, *Leishmania* parasites have been shown to evade protective immune responses by inducing functional $CD8^+$ T cell exhaustion, driving $CD8^+$ T cell anergy and cell death during experimental (Joshi *et al*. 2009) and human (Gautam *et al*. 2014) VL.

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The predominant host protective role of $CD4^+$ T cells during VL is the production of cytokines and chemokines that support granuloma formation and parasite killing. Host defence in the liver is critically mediated by pro-inflammatory Th1-type cytokines, including IL-2 (Murray *et al*. 1987), IL-12 (Ghalib *et al*. 1995), IFN-γ (Squires *et al*. 1989), TNF (Tumang *et al*. 1994), lymphotoxin (LT) (Engwerda *et al*. 2004) and granulocyte/macrophage colony stimulating factor (GM-CSF) (Murray *et al*. 1995*a*). IL-2 is a potent T cell growth factor, which enhances granuloma tissue reactions and parasite clearance during experimental *L. donovani* infection largely through the induction of IFN-γ (Murray *et al*. 1993). Production of IFN-γ by T cells to generate protective responses in the liver is also dependent on IL-12. Control of parasitaemia is lost in the absence of IL-12, and is associated with reduced IFN-γ production and arrested granuloma formation (Murray, 1997). IL-12 may also exert antileishmanial effects independently of IFN-γ, as administration of IL-12 to IFN-γ knockout mice still resulted in parasite killing (Taylor and Murray, 1997). IL-12 is thought to play an important role in the regulation of cellular immune responses in human VL. PBMCs from patients with active VL are unable to produce IFN-γ in response to *Leishmania* antigens *in vitro*; however, the addition of IL-12 restored *in vitro* IFN-γ production (Ghalib *et al*. 1995).

During experimental VL, IFN-γ plays a critical role in the early immune responses that induce tissue granuloma formation and effectively control parasite replication. The neutralization of IFN-γ during infection results in poor cellular assembly of granulomas and an increased parasite burden in the liver (Squires *et al*. 1989). Impaired granuloma formation was also observed in mice deficient in IFN-γ and was associated with an inability of infected Kupffer cells to recruit monocytes and T cells to the liver (Taylor and Murray, 1997). Therapeutic administration of IFN-γ can activate macrophages *in vivo*, but requires the presence of T cells for antileishmanial activity (Murray *et al*. 1995*b*). Experiments in mice showed that administration of IFN-γ increased the efficacy of antimony chemotherapy (Murray, 1990), and IFN-γ has been used as an adjunct therapy for severe or refractory cases of clinical VL (Badaro and Johnson, 1993). Whilst IFN-γ plays a crucial role in the initiation of the granulomatous response early in infection, mice deficient in IFN-γ are capable of reducing liver parasite burdens in the later stages of infection. An early IFN-γ response leads to induction of IL-12 and expression of TNF, and it appears that the late-developing IFN-γ independent antileishmanial mechanism is mediated by TNF (Taylor and Murray, 1997).

TNF is essential for the formation and maturation of the hepatic granuloma response (Murray *et al*. 2000). TNF is required for survival in experimental VL models, as *L. donovani* infection is only fatal in the mouse model in the absence of TNF (Tumang *et al*. 1994). Mice lacking TNF succumb to overwhelming infection after 6 weeks, with accelerated parasite growth in the liver, impaired hepatic granuloma formation and an enhanced inflammatory response (Murray *et al*. 2000). Neutralization of TNF during *L. donovani* infection promotes parasite persistence in the liver, indicating that TNF is required for hepatic resolution (Tumang *et al*. 1994). TNF is produced by infected Kupffer cells throughout the time course of infection (Engwerda *et al*. 1996) and is essential for leucocyte recruitment. LTα, a member of the TNF superfamily of cytokines, is also required for the control of parasite growth in the liver. LTα plays a key role in granuloma formation, facilitating the trafficking of lymphocytes from the perivascular areas of the liver to the infected Kupffer cells (Engwerda *et al*. 2004). The role of LTα in the liver is distinct from that of TNF, as $CD4^+$ T cells that express both TNF and LTα are needed for efficient killing of parasites within assembled granulomas. Other members of the TNF superfamily, such as CD95L, also contribute to host protective immune responses during VL (Alexander *et al*. 2001).

Whilst the emphasis on liver immune defence is generally focused on the production of Th1 cytokines, the co-expression of Th2 cytokines may also contribute to host protective responses. For example, the induction of IL-4 is essential for the formation of mature granulomas and for effective parasite killing (Stager *et al*. 2003). The suppressive effect of immunoregulatory cytokines may limit inflammatory tissue damage in the liver, but generally these cytokines down-regulate critical antileishmanial responses, particularly those dependent on IFN-γ. The production of TGF-β (Wilson *et al*. 1998), IL-6 (Murray, 2008), IL-10 (Murphy *et al*. 2001), IL-27 (Rosas *et al*. 2006) and IL-33 (Rostan *et al*. 2013) impairs effective control of parasite growth in the liver. Mice deficient in IL-6 showed an enhanced ability to control infection with earlier, and more rapid, parasite killing associated with increased levels of circulating IFN-γ and accelerated granuloma formation (Murray, 2008). Expression of IL-33, an IL-1 family member, is increased in the liver during human VL and patients have increased IL-33 serum levels. Lower liver parasite burdens were observed in mice in the absence of IL-33 signalling mice, which was associated with a strong induction of IFN- γ and IL-12 (Rostan *et al*. 2013).

IL-10 is a critical component of the host immune response that inhibits resistance to VL and promotes disease progression. Human VL disease is strongly associated with increased production of IL-10 in a variety of clinical settings and elevated IL-10 levels correlate with the development of pathology (Ghalib *et al*. 1993). The absence of IL-10 leads to enhanced

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resistance to experimental VL infections in mice (Murphy *et al*. 2001). IL-10 has multiple effects on the immune system and suppresses the production of key cytokines, IL-12 and IFN-γ (Murphy *et al*. 2001; Murray *et al*. 2003). Regulation of cellular immune responses by IL-10 includes the suppression of macrophage activation (Bogdan *et al*. 1991) and impaired intracellular killing of *Leishmania* (Bhattacharyya *et al*. 2001). While there are multiple cellular sources of IL-10 during VL infection, a population of Th1-like $CD4^+$ T cells that make IL-10 has been associated with disease progression (Stager *et al*. 2006). Conventional DCs that make both IL-10 and IL-27 can induce the production of IL-10 from effector Th1-like $CD4^+$ T cells and enhance immunopathology (Owens *et al*. 2012).

NK cells and NKT cells participate in the early innate immune responses in the liver and contribute to the control of parasitaemia (Kirkpatrick *et al*. 1985; Svensson *et al*. 2005). CD1d-dependent activation of NKT cells occurs during *L. donovani* infection and these cells also respond with a rapid production of IFN-γ. CD1d-deficient mice show an increased susceptibility to parasitism (Amprey *et al*. 2004). During infection, Kupffer cells can activate invariant NKT (iNKT) cells by engagement of CD47 (Beattie *et al*. 2010*b*) and iNKT cells are essential for regulating chemokines, such as CXCL10 (Svensson *et al*. 2005). There is increasing interest in the development of therapies that enhance iNKT cell function during visceral infection to direct early immunity towards the establishment of a protective host response. However, activation of iNKT cells using the glycolipid antigen α-galactosylceramide (α-GalCer) prior to infection did not show any beneficial effect during VL. Moreover, α-GalCer administered during an established VL infection exacerbated hepatic disease, and was associated with a decrease in IFN-γ-producing $CD8^+$ T cells (Stanley *et al*. 2008*b*). Thus, it appears that iNKT cell activation by α-GalCer hinders disease resolution in the liver, and therapies aimed at modulating NKT cell function may be of limited clinical use.

VL and the spleen: suppression and susceptibility

The spleen is a major organ for the induction of immune responses to infection, and also a site for the killing of parasites during VL. However, prevalent clinical features of human VL include splenomegaly and suppression of antigen-specific immune responses (Zijlstra and el-Hassan, 2001). This immunopathology is recapitulated in experimental murine models where splenomegaly is associated with the persistence of parasites and remodelling of the lymphoid tissue (Polley *et al*. 2005). The kinetics of experimental VL infection display a distinct organ-specific pattern: the liver displays an acute resolving infection, attributed to effective granuloma tissue responses, while VL parasites persist in the spleen resulting in a chronic, unresolved state of infection.

The spleen is a highly organized secondary lymphoid organ, consisting of a specialized marginal zone (MZ), which separates the red pulp and white pulp region. The macrophages in the MZ, the marginal metallophilic macrophages (MMMs) and the MZ macrophages (MZMs), are the main phagocytic cell populations responsible for the clearance of parasites during experimental *L. donovani* infection. The antileishmanial activity of these specialized splenic macrophages is dependent on interferon regulatory factor-7 (IRF-7) (Phillips *et al*. 2010).

Acute immune responses generated in the spleen play a key role in the control of *L. donovani* parasites in the liver during the early phase of infection. The spleen is an important site for DC priming, and DCs are the critical source of early IL-12 following VL infection (Gorak *et al*. 1998). A transient and rapid burst in IL-12 has been observed as early as 5 h post-infection (Ato *et al*. 2006) and is a crucial event for the generation of effective antiparasitic immunity (Engwerda *et al*. 1998). Vascular cell adhesion molecule-1 (*VCAM*-1) and its ligand very late antigen-4 (*VLA*-4) are involved in the initiation of early IL-12 secretion from DCs. Blockade of VCAM-1 or VLA-4 suppressed the production of IL-12 by splenic DCs and reduced parasite-specific T cell responses in the spleen. This was also associated with lower levels of IFN-γ, TNF and NO production in the liver and significantly higher liver parasite burdens (Stanley *et al*. 2008*a*). Migratory DCs may directly phagocytose parasites; however, it is most likely that splenic DCs acquire antigen and are activated by infected macrophages in the MZ. Upon activation DCs migrate to the T cell areas in the peri-arteriolar lymphoid sheets (PALS) and IL-12-producing DCs are observed in the T cell area of the spleen during VL infection. The production of IL-12 by DCs is essential for the activation of effector T cell populations, and the total $CD4^+$ T cell population in the spleen is expanded during experimental infection (Polley *et al*. 2005). T cells are the dominant leucocyte population in the spleen of VL patients, as compared with normal healthy control aspirates that show a predominance of B cells (Nylen *et al*. 2007).

Chemokine-dependent encounters between DCs and T cells in the spleen are crucial for effective responses to *L. donovani* infection. Mice deficient in CCL19 and CCL21 show impaired DC migration in the spleen and a decreased production of IL-12 during *L. donovani* infection. These defects in early DC activation in the spleen were associated with reduced migration of effector T cells to the liver and impaired granuloma formation (Ato *et al*. 2006). Exogenous administration of IP-10 restores T cell proliferative capacity, leading to decreased parasite burdens in the liver and spleen. IP-10 treatment

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during experimental VL induced strong expression of iNOS2 and mediated parasitic killing through increased NO synthesis (Gupta *et al*. 2009). Together the data demonstrate the importance of chemokines in promoting early DC and $CD4^+$ T cell interactions in the spleen and inducing protective immunity against *L. donovani*.

Neutrophils may also play a protective role in the acute response in the spleen, as the absence of neutrophils results in a decrease in IFN-γ-producing $CD4^+$ and $CD8^+$ T cells and an enhanced parasite burden in the spleen. This antileishmanial effect appeared to be specific to the spleen as the absence of neutrophils had only minor effects on parasite growth in the liver (McFarlane *et al*. 2008). Neutrophils do not appear to play a significant role in the chronic stage of infection as long-term administration of neutrophil-depleting antibody does not significantly increase parasite burdens in either organ (Rousseau *et al*. 2001).

During experimental *L. donovani* infection in mice, no resolution of infection occurs in the spleen and animals maintain chronic parasite burdens in this tissue. There is evidence of profound immune dysfunction in the spleen with an impairment of antigen-specific T cell responses, increased T cell apoptosis (Alexander *et al*. 2001) and the production of regulatory cytokines, such as IL-10 (Stager *et al*. 2006) and TGF-β (Wilson *et al*. 2002). NK cells are negative regulators of cell-mediated immunity in the spleen and show enhanced secretion of IL-10 in the chronic phase of infection (Maroof *et al*. 2008). Marginal zone B cells in the spleen have been shown to suppress antigen-specific CD8 and CD4T cell responses during the early stages of VL (Bankoti *et al*. 2012). B cells also suppress NK cells and inhibit the generation of effector memory CD8T cells after *L. donovani* infection (Bankoti *et al*. 2012).

Whilst the production of TNF is crucial for the induction and maintenance of host protective responses in the liver, TNF is a key mediator of pathology in the chronically infected spleen. During the later stages of VL, high numbers of TNF-producing cells are present in the spleen and TNF production is observed in both the red and white pulp regions (Engwerda *et al*. 1998). TNF is the principal cytokine responsible for the breakdown of splenic architecture following experimental *L. donovani* infection, contributing to remodelling of the MZ (Engwerda *et al*. 2002) and the loss of stromal cells from the PALS (Ato *et al*. 2002). Infection-induced re-modelling of the MZ is associated with a dramatic and rapid loss of MZMs, whilst MMMs undergo repositioning within the sinus. In mice lacking TNF or mice treated with TNF neutralizing monoclonal antibodies, MZMs were preserved, indicating that the loss of MZMs is a TNF-dependent process (Engwerda *et al*. 2002). Evidence for the role of TNF in disease pathogenesis in human VL arises from studies of TNF polymorphisms. A study in northern Brazil examined polymorphisms in the *TNFA* promoter (TNF1 and TNF2 alleles) in neighbourhoods with ongoing transmission. The presence of the TNF2 allele was more frequent in individuals with progressive disease, whilst the TNF1 allele was associated with asymptomatic infection. The presence of the TNF2 susceptibility allele was associated with higher levels of serum TNF as compared with the TNF1 allele, suggesting that increased TNF is involved in the progression of human VL (Karplus *et al*. 2002).

The activation of B cells is a key clinical indicator of VL infection, with patients displaying polyclonal hypergammaglobulinaemia (Ghose *et al*. 1980), polyclonal B cell activation and increased circulating immune complexes. The role of immunoglobulins during VL is controversial, as large amounts of immunoglobulins to both parasite-specific and non-specific antigens are produced during infection, including auto-antibodies (Galvao-Castro *et al*. 1984). These immunoglobulins are not thought to be protective as elevated levels of total antibody correlate with disease pathology (Anam *et al*. 1999) and have been implicated in the development of anaemia (Pontes De Carvalho *et al*. 1986) and auto-immunity (Galvao-Castro *et al*. 1984). Experimental models of VL using B cell-deficient mice have demonstrated that B cells are not required for the control of parasite burdens. Additionally the re-constitution of mice with immunoglobulin leads to disease exacerbation through complement activation and signalling (Deak *et al*. 2010). However, B cells may have some regulatory role to play in suppressing immunopathology, as the absence of B cells leads to sustained neutrophil-mediated pathology of the liver (Smelt *et al*. 2000).

Follicular DCs (FDCs), a resident stromal cell population, play a key role in the organization of lymphoid follicles in the spleen and facilitate the germinal centre (GC) reaction. FDCs are involved in B cell activation, proliferation and maturation through presentation of antigen and production of regulatory signals such as chemokines. During the chronic stage of *L. donovani* infection, the FDC network is destroyed and there is a concomitant loss of GC (Smelt *et al*. 1997). The complete absence of FDCs is associated with the infiltration of heavily parasitized macrophages into the splenic white pulp regions. It has been hypothesized that the B cell function may become dysregulated in the absence of FDCs and thus the loss of FDCs may contribute to the hypergammaglobulinaemia observed during VL.

Impaired DC migration plays a major role in the pathogenesis of VL and alterations to stromal cell populations directly contribute to immunosuppression during the chronic stage of *L. donovani* infection. Splenic DCs increase in number during the chronic phase of infection but fail to migrate from the MZ

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to the PALS. This impaired migration is due to a disruption in the fibroblastic reticular cell (FRC) network that guides T cell and DC migration in the T cell zone of the spleen. The changes to the splenic FRC network are due to a TNF-dependent loss of podoplanin $(gp38)^+$ stromal cells (Ato *et al*. 2002). Down-regulation of CCR7 from the DC cell surface also impairs DC migration in the spleen during VL. TNF is also implicated in this process, as enhanced levels of TNF increase IL-10 production, and IL-10 directly induces the loss of CCR7 expression on the DC surface (Ato *et al*. 2002). A potential therapeutic role for DCs has been proposed, as adoptive transfer experiments show that administration of *in vitro* activated DCs can reduce parasite burdens in the spleen. The efficacy of DC therapy relies on both IL-12 and IL-6, with IL-6 thought to suppress the expansion of IL-10-producing T cells (Stager *et al*. 2006). However, recent studies demonstrate that some populations of DCs may contribute to splenic pathology, as targeted deletion of DCs during the established phase of infection improved disease resolution (Owens *et al*. 2012).

Interventions that preserve splenic structure during VL have been shown to improve the host response to chemotherapy by enhancing parasite killing. Treatment of experimental VL with receptor tyrosine kinase inhibitors reduced splenomegaly, prevented vascular remodelling and restored the integrity of the microarchitecture of the spleen. Importantly, the maintenance of splenic architecture during infection improved the host response to drug treatment, with a 10-fold reduction in the amount of antimony required to clear infection (Dalton *et al*. 2010).

CONCLUDING REMARKS

Leishmania parasites activate the innate and adaptive arms of the immune system, and it is clear that a coordinated network of responses is required for effective immune-mediated parasite clearance. The timing of key chemokine and cytokine responses is essential and involves a tight regulation of cellular populations of the immune system. However, *Leishmania* parasites have developed numerous mechanisms to prevent development of immunity and promote resistance. These include induction of immunosuppressive cytokines, interruption of signalling pathways in macrophages and dendritic cells and induction of regulatory T cells. Resistance to infection is also enhanced by the negative regulatory role of NK cells in chronic disease and the presence of Th2 cell-attracting chemokines in lesions. All these mechanisms assist the parasite in avoiding immune clearance and increase the chances of successful transmission of *Leishmania* parasites to a new host. Understanding the complexity of immune responses involved in *Leishmania* disease pathogenesis and protection offers hope for development of effective vaccines and immunotherapeutic interventions.

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