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The immunopathology of experimental visceral leishmaniasis

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Summary: Experimental murine infection with the parasites that cause human visceral leishmaniasis (VL) results in the establishment of infection in the liver, spleen, and bone marrow. In most strains of mice, parasites are eventually cleared from the liver, and hepatic resistance to infection results from a coordinated host response involving a broad range of effector and regulatory pathways targeted within defined tissue structures called granulomas. In contrast, parasites persist in the spleen and bone marrow by mechanisms that are less well understood. Parasite persistence is accompanied by the failure of granuloma formation and by a variety of pathologic changes, including splenomegaly, disruption of lymphoid tissue microarchitecture, and enhanced hematopoietic activity. Here, we review the salient features of these distinct tissue responses and highlight the varied roles that cytokines of the tumor necrosis factor family play in immunity to this infection. In addition, we also discuss recent studies aimed at understanding how splenomegaly affects the survival and function of memory cells specific for heterologous antigens, an issue of considerable importance for our understanding of the disease-associated increase in secondary infections characteristic of human VL.

Introduction

Visceral leishmaniasis (VL) is a potentially fatal human disease caused by the intracellular protozoan parasites *Leishmania donovani* and *Leishmania infantum* (*chagasi*) (1). Parasites can be found in mononuclear phagocytic cells throughout the viscera, though the spleen and liver are the major sites of disease (1, 2). VL is responsible for significant morbidity and mortality in the developing world, particularly in India, Sudan, Nepal, Bangladesh, and Brazil (3). Many deaths associated with this disease are caused by secondary infections that arise as a result of the immunocompromised status of VL patients (1). The mechanisms preventing clearance of this infection and underlying the predisposition of VL patients to secondary infections are not fully understood. At present, there is no effective vaccine available. In addition, the major drug used to treat infected people, pentavalent antimony, has been in use for over 50 years, and significant parasite resistance has now been reported as well as increased drug toxicity in patients caused by the need for longer treatment periods (3).

One of the major hurdles for developing effective prophylactic or therapeutic vaccines as well as safer and more effective drugs has been a limited understanding of the precise immune mechanisms required for the priming and maintenance of T-cell responses and the appropriate delivery of effector function(s) within the varied tissue microenvironments that contain infected macrophages. Because of the intrusive techniques that would be required to analyze such responses in VL patients, our current understanding of the host immune response during VL largely derives from studies performed in mice.

The mouse model of VL

In most laboratories, experimental VL is initiated by intravenous injection of amastigotes of either *L. donovani* or *L. infantum* (*chagasi*). The outcome of such infections in mice has a clear genetic basis. Early amastigote replication in tissue macrophages is regulated by the phagosomal proton-cation antiporter encoded by the *Slc11a1* gene (formerly *Nramp1* or *Lsh/Bcg/Ity*) (4–6). Whereas early parasite growth can be controlled in *Slc11a1* wildtype mice (e.g. CBA), it is unrestrained in *Slc11a1* mutant mice (including both BALB/c and C57BL/6 strains), and by 14 days post-infection (p.i.), *Slc11a1* mutant mouse strains contain 50–100-fold more amastigotes in their livers than wildtype counterparts (7, 8). Expression of this natural resistance is independent of T cells and other aspects of acquired immunity. In contrast to mice, where the $(\text{Gly}\rightarrow\text{Asp})_{169}$ substitution associated with *Slc11a1* mutant strains confers a functional 'null' phenotype (9), polymorphism in the human *SLC11A1* gene is confined to promoter regions (4). In a recent study, where several chromosomal regions containing candidate disease resistance genes were typed, linkage was established between VL and the 5′ (CA) repeat polymorphism of the *SLC11A1* promoter (10).

Although most *Slc11a1* mutant mice eventually control their hepatic infection, the rate and effectiveness of this control is determined largely by major histocompatibility complex (MHC) haplotype (6, 11), indicating an important role for acquired immune responses. The mechanisms underlying this host resistance to both *L. donovani* and *L. infantum* infection have been studied most extensively in the liver. Because $CD4^+$ and $CD8^+$ T cells are both required for optimal resistance in normal mice, resistance is as expected absent in nude, severe combined immunodeficiency disease (SCID), or recombination activating gene $(Rag)^{-/-}$ mice, and reconstitution experiments suggest that both populations of T cells can effectively transfer resistance, with varying degrees of efficacy (12–17). Association between rate of cure and relative T-helper 1 (Th1) : Th2 bias has been difficult to detect (18). Most *Slc11a1* mutant mouse strains have a mixed T-cell cytokine profile, apparent throughout the infection (18, 19). By enzyme-linked immunospot assay (ELISPOT) analysis, interferon γ (IFNγ)- and interleukin 4 (IL-4)-producing cells are found at a ratio of approximately 2–3:1, with only minor variations over time and in different organs.

Hepatic resistance correlates well with the generation of reactive oxygen and reactive nitrogen intermediates. In early stages of infection (to day 14 p.i), both play significant roles in containing parasite growth, as determined by studies in $\text{phox}^{-/-}$ and nitric oxide synthase $(\text{NOS})\text{-}2^{-/-}$ mice (20), and this significance may relate to T-cell-dependent recruitment of both neutrophils and monocytes at these early times (21, 22). During the later phase of infection, when hepatic resistance is expressed by declining amastigote numbers, NOS2 gene regulation appears to play the more dominant role, and the generation of NO mainly reflects T-cell-dependent macrophage activation (20). Although efficiency of macrophage activation may reflect cytokine-mediated cross-regulation at the level of Th-cell differentiation, cross-regulation at the level of NOS2 gene expression in macrophages themselves appears to play a dominant role, with production of the inhibitors of NOS2 gene expression, IL-10, and transforming growth factor β (TGFβ) also correlating well with reduced parasite killing (23–28). These two counter-protective cytokines may be temporally regulated, with TGFβ appearing rather late in infection compared to IL-10. To date, the range of cellular sources of these cytokines in VL has not been fully characterized, though it is clear that T cells contribute both to TGFβ production (24) and to IL-10 production (Maroof, unpublished data).

The cardinal histopathological feature of hepatic resistance to visceralizing species of *Leishmania* is the development of granulomas. Diversity of granuloma structure and function in models of experimental VL has been extensively reviewed (29), and many of the cellular and molecular components of acquired immunity necessary for the formation, maintenance, and effector function of granulomas have been characterized through the use of gene-targeted mice or *in vivo* administration of neutralizing or depleting monoclonal antibodies. Granuloma development or 'maturation' can be readily staged by histologic criteria, and such analysis on individual infected foci clearly indicates that this process occurs in an asynchronous manner. Mature granulomas, showing extensive mononuclear cell cuffing, can readily be seen alongside Kupffer cells that harbor intracellular amastigotes but that appear to have

failed to trigger an inflammatory response. The mechanistic basis of such asynchrony is not well understood, but it may reflect intrinsic differences in Kupffer cell populations with respect to chemokine production, indirect effects due to varying amastigote load, or a limitation imposed by the frequency of antigen-specific T cells available for recruitment to the liver (30).

Immune effector mechanisms operating in the hepatic environment appear to be relatively easy to manipulate experimentally. For example, administration of neutralizing monoclonal antibodies to cytokines or direct cytokine administration often has rapid effects on rate of cure (apparent over a 7-day time period). Unlike models of cutaneous leishmaniasis where only a narrow window of opportunity exists to manipulate cytokine cascades (31), in experimental VL, manipulation at any time during the course of infection may have profound effects (29, 32). Similarly, immunotherapy based on manipulating costimulatory pathways, including CD40-CD40L, OX40-OX40L, and cytotoxic T-lymphocyte antigen (CTLA)-4–CD80/86 interactions (33–36), has a clear beneficial effect on hepatic resistance, often approaching that of conventional chemotherapy. Collectively, these data suggest that the immune response to VL may be continually in a state of flux, with T-cell responses being induced and lost in rapid succession, rather than the effector response being a feature of long-lived T cells primed early during infection. A relatively high rate of T-cell apoptosis in mice with VL supports such a possibility (12).

In contrast to the self-limiting infection seen in the liver of most *Slc11a1* mutant mouse strains, these experimental VL mice invariably maintain readily detectable numbers of amastigotes in their spleens, usually for life. The spleen becomes massively enlarged (up to 15% of body weight within 6–8 weeks of infection) (37). Although some degree of effector response is present, evident by comparing immunocompetent and immunodeficient strains and from the effects of neutralization of IL-12 (38), host resistance mechanisms induced during natural infection fail to eliminate parasites to a level where normal tissue homeostasis is restored. Furthermore, the spleen is often refractory to many of the interventions which successfully alter resistance in the hepatic environment, e.g. costimulation-based therapy (Zubairi, unpublished data).

In the spleen and indeed in the bone marrow, where parasite growth and host resistance patterns closely resemble the spleen (39), granuloma formation is markedly delayed or absent. If present, granulomas do not proceed past immature collections of coalescing macrophages, found in the red and white pulp. The basis for this organ-specific failure in the development of granulomatous reactions and local host protection remains a major gap in the understanding of VL (13). The remainder of this review highlights recent advances in our understanding of the immunopathology associated with experimental VL, the impact that pathology has on local immune regulation, and the multiple roles that tumor necrosis factor (TNF) family cytokines may play in these events.

The multifaceted role of TNF

TNF is a prototypic pro-inflammatory cytokine. It has multiple cellular sources, of both hematopoietic and nonhematopoietic lineage and is a key feature of the innate immune response to many pathogens (40, 41). Similar to the situation observed in other infectious diseases, notably malaria (42–44), TNF has a variety of roles in experimental VL. Some benefit host resistance, while others mediate host pathology. In this section, the influence of TNF and other TNF family cytokines at various stages in the progression of VL is discussed (Fig. 1).

An essential role for TNF family cytokines in the development of lymphoid tissues

Appropriate development of the intricate microarchitecture of the spleen and other lymphoid tissues requires the coordinate function of members of the TNF family of cytokines. Mice deficient in TNF, lymphotoxin α (LTα), LTβ, or corresponding receptors all have profound, though distinct, changes to their spleen and other lymphoid tissues (41, 45–49). TNF$^{-/-}$ mice have flattened Peyer's patches with loss of T-cell and B-cell segregation, but lymph nodes are relatively unaffected by loss of this cytokine (49). In the spleen, TNF$^{-/-}$ mice also show a loss of segregation between B and T cells and lack primary follicles, the endothelial cells of the marginal sinus

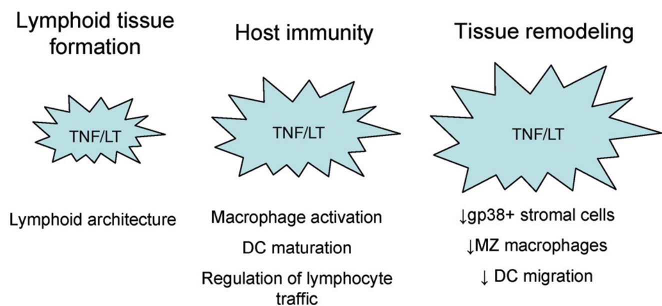

Fig. 1. The varied roles of tumor necrosis factor (TNF) family cytokines in immune responses to *Leishmania donovani*. TNF and lymphotoxin (LT) play multiple roles in the organization of resting lymphoid tissue architecture during development, are involved in multiple features of the innate and adaptive response to infection, and, when in excess, play roles in destructive tissue remodeling and aberrant dendritic cell function.

have reduced mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression, and marginal metalophilic macrophages (MMM) are entirely lost (41, 47, 49). A comparison between TNF$^{-/-}$ mice and mice in which there has been targeted replacement of the *TNF* gene with a version encoding only membrane-bound TNF (memTNF) indicates that memTNF partially compensates for the loss of TNF. Mice expressing memTNF have increased numbers of MMM (though not to wildtype levels) and there is substantial restoration of MAdCAM-1 expression. However, memTNF cannot compensate for the loss of soluble TNF in respect to primary follicle formation. Similarly, memTNF restores T-cell and B-cell segregation but not follicle formation in the Peyer's patch (50). The impact of TNF deficiency on lymphoid tissue structure is believed to reflect alteration in chemokine-dependent homing of lymphocytes into this organ, and the partial restoration of splenic architecture seen in memTNF compared to TNF$^{-/-}$ mice reflects the capacity of memTNF to restore endogenous levels of a variety of chemokines to those found in wildtype mice (41, 50). In contrast to the selective defects in the MZ of TNF$^{-/-}$ mice, LTα-deficient mice lack the entire marginal zone (MZ), as well as exhibiting a lack of peripheral lymph nodes (45, 51). In addition to roles in organogenesis, LT$\alpha_1\beta_2$ but not TNF plays a role in the steady state maintenance of spleen structure, as evident from the effects of administration of monoclonal antibodies or Ig fusion constructs that interfere with LTβR signaling (52). Thus, members of the TNF family of cytokines play pivotal roles in the organization of lymphoid tissue and hence the innate capacity of the host to mount an immune response to *L. donovani* infection. Following infection, however, these cytokines play even more diverse roles.

TNF expression at the single cell level

Infection of macrophages is a key event in the establishment of infection by a diverse range of intracellular pathogens. Although the receptors that mediate recognition are extremely diverse, as is the capacity to engage various Toll-like receptors (TLRs) (53), uptake of most pathogens by macrophages leads to the rapid induction of TNF. Both mRNA accumulation and TNF secretion are usually readily apparent within 1 or 2 h of microbial exposure *in vitro* or *in vivo* (54, 55). In contrast, exposure of macrophages to *L. donovani* amastigotes or promastigotes *in vitro* and *in vivo* results in limited TNF production (56, 57). Detailed analysis of a variety of *Leishmania* species, differing in the structure of their major glycolipids, suggests that those that cause visceral disease are particularly adept at bypassing this proinflammatory host response. It has been proposed that this response is largely determined by the variable nature of the surface-expressed lipophosphoglycan, which in *L. donovani* is unsubstituted with the side chains associated with most other species of *Leishmania* (58–60).

In mice, TNF is not initially detected in macrophages of the MZ, the primary target of infection of intravenously administered amastigotes of *L. donovani*. However, TNF expression can be readily observed by 3 days later both on infected and on uninfected MZ macrophages, most likely representing the requirement for IFNγ-mediated priming of macrophages for TNF secretion (57). Furthermore, the observation that TNF is readily observed at this time in both infected and uninfected macrophages suggests that amastigotes do not actively inhibit the capacity of macrophages to produce this cytokine, as they so effectively do for IL-12 (56–58, 61). Likewise, TNF expression is readily observed on infected Kupffer cells in the liver at both early and late stages of the hepatic response, but not in Kupffer cells of infected SCID mice at any stage of infection (62).

TNF, granuloma formation, and resistance to hepatic infection

The role played by TNF at later stages of host resistance in the liver is complex and has been studied using gene-targeted mice, antibody neutralization, and by administration of exogenous TNF. Early studies indicated that TNF was critical to host resistance (63). Murray and colleagues (64) subsequently demonstrated that TNF$^{-/-}$ mice on a mixed B6/129 background were highly susceptible to infection and died within 2 months, one of the rare situations in which *L. donovani* infection of mice progresses to a fatal outcome. The essential role for TNF in host resistance and host survival has recently been confirmed by us using B6.TNF$^{-/-}$ mice (Engwerda *et al.*, manuscript submitted). As lack of TNF also gives rise to changes in lymphoid tissue architecture (as noted above), it was important to rule out the possibility that such effects indirectly lead to loss of hepatic resistance, e.g. due to poor T-cell priming or cellular migration rather than TNF itself being a mediator of the effector response. To this end, we also generated radiation bone marrow chimeras, transferring bone marrow from TNF$^{-/-}$ mice into wildtype B6 mice recipients. In addition, study of such chimeras has provided a means to differentiate the relative contribution that production of TNF by hematopoietic and nonhematopoietic cells makes to various aspects of the host response. B6.TNF$^{-/-}\rightarrow$B6.CD45.1 chimeric mice succumb to infection and die with similar kinetics to

B6.TNF$^{-/-}$ mice, whereas control B6→B6.CD45.1 chimeras resist infection and survive. Thus, hematopoietic cells are a critical source of TNF involved in host protection. We also employed an adoptive transfer model, whereby host resistance can be reconstituted in B6.RAG1$^{-/-}$ mice using CD4^{+} T cells (12). Whereas B6-derived CD4^{+} T cells were able to transfer protection to RAG$^{-/-}$ recipients, CD4^{+} T cells from TNF$^{-/-}$ mice could not do so (Engwerda *et al.*, manuscript submitted).

TNF is also essential for the rapid, optimal maturation of hepatic granulomas (64), though initial studies using neutralizing antisera suggested otherwise (63). In B6/129.TNF$^{-/-}$ mice, rapid amastigote growth initially occurs in the complete absence of granuloma formation. However, later in infection, granuloma assembly abruptly begins and parasite growth starts to come under control. This late antiparasite response, however, is not beneficial, as all TNF$^{-/-}$ mice die shortly afterward with fulminant hepatic necrosis (64). Although TNF is required for optimal granuloma formation, in the adoptive transfer model described above, production of TNF by CD4^{+} lymphocytes is not essential for granuloma development (Engwerda *et al.*, manuscript submitted).

The specific cause of death in TNF$^{-/-}$ mice is unknown. Histologically, there is extensive hepatic necrosis and neutrophil infiltration. Given that TNF$^{-/-}$ mice die with similar symptoms following a wide range of infectious and non-infectious stimuli (65), death would not appear due to the presence of *Leishmania* parasites *per se*. The hepatic tissue burden observed in TNF$^{-/-}$ mice can clearly be matched or even exceeded in other cytokine gene-deficient mice and in RAG mice, yet death does not occur. Circumstantial evidence suggests that this fatal outcome in TNF$^{-/-}$ mice may relate to a requirement for TNF to induce high levels of host protective IL-10. IL-10 is well known to be essential to prevent excess tissue damage following induction of Th1 type T-cell responses (66, 67), and IL-10 mRNA accumulation is markedly decreased in TNF-deficient mice infected with *L. donovani* (68).

Collectively, the data discussed above implicate TNF as a major beneficial mediator of antileishmanial immunity in the liver and of the host tissue response. However, it is of interest that when Murray and colleagues (63) administered recombinant TNF in high concentrations by osmotic pump, a reduction in host resistance was observed and granuloma development was also impeded. Unfortunately, the long-term consequences of TNF administration are unknown. Nevertheless, this report indicates that in the liver of mice, tight regulation of TNF production may be an essential feature of the response of this organ to infection.

Role of LTα in VL

We have recently also evaluated the role of the TNF-related cytokine LTα in control of hepatic infection with *L. donovani*, given that some of the functions of these cytokines may be shared and that LTα has been implicated in the pathogenesis of other infections (44, 66, 67, 69–72). As with TNF$^{-/-}$ mice, the marked structural defects in lymphoid tissue organization that are observed in LTα-deficient mice require that a comparison between conventional gene-targeted mice and radiation chimeras is made. Our recent studies indicate that TNF and LTα both have important and in some cases noncompensatory roles in the development of resistance to hepatic infection. First, LTα$^{-/-}$ mice and LTα$^{-/-}$→B6.CD45R.1 chimeras are more susceptible to early *L. donovani* infection. The increase in parasite burden at day 14 is similar to that seen in TNF$^{-/-}$ or TNF$^{-/-}$→B6.CD45R.1 mice. Second, adoptive transfer of LTα-deficient CD4^{+} T cells into RAG mice failed to enhance protection, unlike transfer of wildtype CD4^{+} T cells. Thus, both LTα and TNF are required for optimal control of early hepatic infection, and neither cytokine can compensate for the loss of the other. Third and in marked contrast to TNF$^{-/-}$ mice or TNF$^{-/-}$→B6 chimeras, LTα$^{-/-}$ mice and LTα→B6 mice acquire later resistance to infection, clear their parasites, and survive long-term. Thus, TNF but not LTα is required to protect against an otherwise lethal inflammatory response. Little is known about the regulation of IL-10 by LTα, though these data might suggest a further function that discriminates LTα and TNF. Fourth, although early granuloma formation is inhibited in the absence of either TNF or LTα, the histological picture is somewhat different. Whereas TNF-deficient mice accumulate few leukocytes in the liver, LTα-deficient mice have intense periportal leukocyte infiltration, but these cells apparently fail to migrate deeper into the sinusoidal area. The distribution of leukocytes in LTα-deficient mice is reflected in the expression of vascular cellular adhesion molecule-1 (VCAM-1), which is known to be regulated by LTα (73). Thus in B6 and TNF-deficient mice, VCAM-1 expression is evident throughout the sinusoidal area, whereas in LTα-deficient mice it is restricted to periportal regions. Furthermore, VCAM-1 expression was mediated by LTα derived from nonhematopoietic cells (most likely the sinusoidal endothelial cells), as evident from the intact VCAM-1 expression on the sinusoids of infected LTα$^{-/-}$→B6.CD45.1 chimeric mice (Engwerda *et al.*, manuscript submitted).

TNF as a mediator of tissue remodeling in the spleen

As described above, splenomegaly accompanies parasite persistence in the spleen of mice infected with *L. donovani*.

Histological examination of these enlarged spleens show similar changes to that observed in lymphoid tissues taken from fatal cases of human VL (2). The white pulp is disorganized and reduced in size, there is hypertrophy of the red pulp, and heavily infected macrophages are found in multiple locations. Recent studies aimed at dissecting the basis of immunopathology in the spleen of *L. donovani*-infected mice have proved particularly informative with regard to understanding the immune dysfunction noted during this infection. Defects observed in the spleen, which begin to become apparent as parasite burden increases at around days 14–21 of infection and as splenomegaly ensues, include the following: (i) loss of gp38^{+} stromal cells, which normally constitute part of the scaffold along which T cells, and dendritic cells (DCs) enter the periarteriolar sheath and which are a major source of the constitutive chemokines CCL19 and CCL21 (68), (ii) MZ remodeling (74), (iii) altered migration of CD11c^{hi} DCs (68), and (iv) loss of follicular dendritic cell networks (37). Some of these changes are shown in *Fig.* 2, and they are discussed in more detail below.

At this time, TNF expression is also dramatically elevated. High levels of cytokine are readily observed by immunohistochemistry, and the frequency of spleen cells spontaneously producing TNF, as detected by ELISPOT assay, increases from $<$1:30000 in naïve mice to approximately 1:450 in mice infected for 28 days (38).

The rodent MZ has a well-defined microarchitecture, and it comprises a marginal sinus, two unique populations of macrophages (MMM and MZM), unique B cells involved in T-independent responses, and a variety of trafficking populations including DCs and T cells (75). The MZ plays significant roles in the clearance of blood-borne pathogens, including *L. donovani* (57), the initiation of T-independent B-cell responses (76, 77), and in regulating T-cell traffic into the periarterial lymphatic sheath (PALS) (78). A major feature of the MZ remodeling that occurs during the progression of experimental VL is the loss of MZM. Unlike the problems associated with tracking cells *in vivo* under inflammatory conditions, where cell-specific antigens may be downregulated or their specificity in the naïve state may be blurred by inflammatory cytokine regulation, the uptake of Indian ink, a solution of colloidal carbon, provides a stable long-term marker of MZM. To understand why MZM might be lost from the MZ of infected mice, we first needed to understand why such cells are positioned as they are in normal uninfected mice. Little is known about this aspect of MZM biology. Given that optimal positioning of many cell types is dependent on chemokine signaling, we examined MZM in a range of mice with chemokine defects. Mice with the spontaneously arising *plt* mutation (*plt/plt* mice) lack the genes encoding CCL19 and the serine isoform of CCL21 (CCL21-ser). As previously described (79, 80), these mice have reduced numbers of T cells and DCs in the white pulp, coincident with a role for these chemokines in regulating migration to this site. However, we noted that the number of MZM was also dramatically reduced in *plt/plt* mice. Although MZM may interact with MZ B cells, no evident changes in MZ B-cell number or positioning were seen in *plt/plt* mice. Furthermore, we were able to show in normal mice that MZM are responsive to CCL21 and CCL19 chemokine gradients, that CCL21-ser is also expressed by endothelium in the MZ, probably in terminal arterioles, and that *in vivo* blockade of Gi protein receptor-coupled signaling using pertussis toxin could cause migration of MZM out of the MZ and into the red

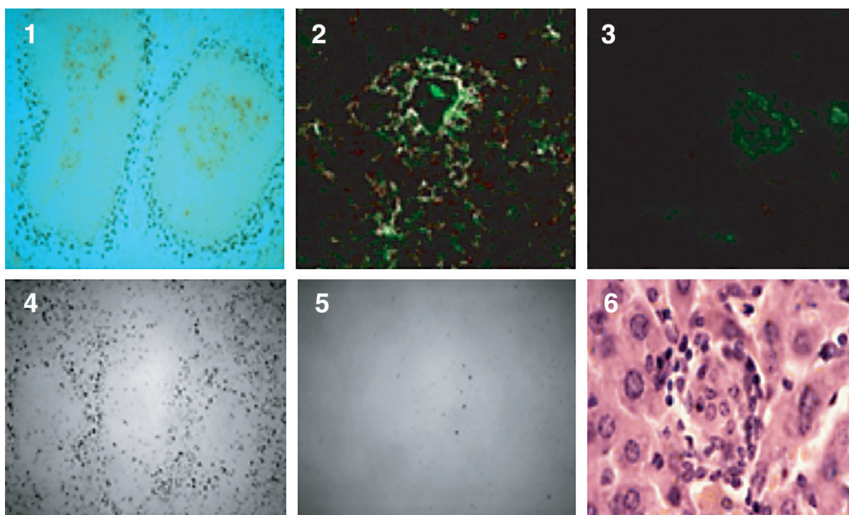

***Fig.* 2. Microenvironmental responses to *L. donovani* infection**. Dendritic cells are stimulated by infection to produce IL-12p40 (brown) in the T cell zone of the normal spleen, whilst most parasites reside in Indian ink^{+} MZM (black) (1). T zone stromal cells (gp38; red) that express CCL21 (green) in the normal spleen (2) are absent from the spleen of chronically infected mice (3). Likewise, MZM (black) in the normal spleen (4) are also lost on chronic infection (5). In contrast to this disruption, hepatic granulomas represent a highly ordered effector structure (6).

pulp (Ato *et al.*, manuscript submitted). These data suggested that CCL21 and CCL19 were intimately involved in positioning of MZM in the normal spleen. The results from this study are consistent with a model whereby the loss of gp38^{+} stromal cells, also seen during *L. donovani* (68), indirectly affects MZM positioning. Further, loss of MZM was far less apparent in TNF$^{-/-}$ mice, and these mice also retained the stromal cells largely responsible for production of CCL19 and CCL21. As yet, the mechanism by which TNF mediates this tissue remodeling is unclear. Metalloproteinase-mediated events may be important, or high local concentrations of TNF may inhibit the expression of CCL21 by stromal cells or endothelial cells in the MZ. Further studies are ongoing to clarify these issues.

In contrast to the effects of TNF-dependent regulation of CCL21 and CCL19 expression on MZM distribution, we could not find evidence using an adoptive transfer model that loss of chemokine secretion from stromal cells in the periarteriolar region directly affected the migration of DCs to this site (68). This result appeared to directly contradict the observed accumulation of DCs in the MZ of chronically infected mice. However, further adoptive transfer experiments, where DCs from infected mice were shown to fail to migrate into the periarteriolar region of normal recipients, indicated that this aberrant DC migration was due to loss of CCR7 expression rather than loss of the chemokine source. This interpretation was confirmed directly by demonstrating reduced staining of DCs from infected mice with a CCL19-Ig fusion protein and by the lack of migration of DCs isolated from infected mice in response to CCL19 and CCL21 *in vitro*. Surprisingly, TNF did not appear in this case to work directly on DCs in infected mice. Instead, TNF provided the *in vivo* stimulus for elevated IL-10 expression and IL-10, rather than TNF, was responsible for downregulation of CCR7 expression on splenic CD11c^{hi} DCs (68).

TNF, splenic architecture, and human VL

TNF expression is markedly upregulated during human VL and may lead to extensive cachexia (1). It is relevant therefore to ask whether any of the above changes observed in the murine spleen as a consequence of TNF action have bearing on human disease. There are clear anatomical differences between the rodent spleen and that of man and other primates (81–83): (i) human T cells do not occur in continuous periarteriolar sheaths around a central arteriole but instead are interrupted by B-cell follicles, which comprise the bulk of the white pulp; (ii) the MZ primarily encloses the follicles. IgM^{+}IgD$^{-/lo}$ MZ B cells occupying the broad inner part of the follicular MZ (iMZ), whereas IgM^{+}IgD^{hi} recirculating B cells occupy the outer follicular MZ (oMZ). T cells and specialized MAdCAM-1^{+}VCAM-1^{+} myofibroblasts lie between the iMZ and the oMZ; (iii) the marginal sinus is absent, as are MMM; and (iv) there is an additional region outside the MZ, the perifollicular zone, where blood enters the open splenic circulation. This region contains sialoadhesin^{+} macrophages surrounding sheathed capillaries and may correspond functionally to the rodent marginal sinus (83). It has also recently been reported that CCL21 may be expressed by fibroblasts found in the perifollicular zone (82).

In spite of these differences, TNF family members appear to play similarly important roles in the maintenance of splenic architecture, as observed in mice. Thus, treatment of cynomolgus monkeys with an LTβR-Ig fusion protein leads to the collapse of the iMZ, such that myofibroblasts lay directly next to the IgD^{+} follicular mantle. VCAM-1 expression is also lost, similar to the outcome of such treatment in mice (83). Thus, at least some of the regulatory controls on splenic microarchitecture are apparently conserved across species. Although the microanatomy of the human spleen appears more highly variable than that of mice, age-dependent effects on structural development can also be observed. Of particular interest, it has recently been shown that lack of MZ development is more often observed in postmortem spleens from children than died of infection or sudden infant death syndrome, compared to control spleens from victims of trauma (84). This study further emphasizes the importance of the MZ for host defense in man. It is tempting to speculate that poor MZ development may be associated with some of the age-dependent effects noted in the epidemiology of human VL.

As far as studies on the histopathology of the spleen in human VL are concerned, detailed descriptions are lacking. Enlargement of the spleen is one of the defining features of VL. There is white pulp atrophy, loss of T cells from the periarteriolar region, and a widespread infiltration of plasma cells and infected macrophages in both the white pulp and red pulp. Lymph nodes, if involved, show follicular destruction, lack of germinal centers, and replacement of the paracortical T cells with parasitized macrophages. Epithelioid granulomas, characteristic of cutaneous leishmaniasis, are not observed. Both the spleen and lymph nodes may show evidence of immune complex deposition (1, 2). There have been no recent immunohistological studies of VL in which cellular phenotype has been characterized using modern immunological markers. Such studies would be highly informative.

That TNF family members are involved in the progression of human VL is also inferred from some but not all genetic studies of TNF polymorphisms. Data from northeastern Brazil suggest that progressive VL is more likely in individuals with the TNF2 allele at −308 of the TNFα promoter. This allele is associated with higher levels of TNFα gene transcription and elevated resting serum TNFα concentrations. In contrast, individuals with the TNF1 allele are more likely to develop asymptomatic infection (85). Other studies have failed, however, to detect such associations (86). There have been no published studies on LTα gene polymorphisms associated with VL, though these have been found for a variety of other chronic human diseases (87–89).

TNF has become a clinically relevant target for the treatment of chronic inflammatory diseases including rheumatoid arthritis and Crohn's disease (90). Although the specter of tuberculosis reactivation may limit use of such therapies in the developing countries (91), other chronic forms of leishmaniasis do appear to benefit from conventional chemotherapy given in combination with anti-TNF drugs (92). Given that effective T-cell immunity underpins the activity of conventional antimonial therapy, further clinical investigations to determine whether the architecture of local lymphoid tissues is restored by TNF blockade would be of great value.

Hematopoiesis in VL

Commensurate with the loss of control of parasite multiplication and the development of the immunopathology described above, hematopoietic activity in the spleen of *L. donovani*-infected mice is also seen to rise (39). Hematopoietic activity can be readily assessed by allowing progenitor cells to give rise to colonies on semisolid agar. In our initial studies, we supplemented the media with erythropoietin, a source of colony-stimulating factors (CSFs) and with stem cell factor (SCF) in order to maximize colony outgrowth. Colonies can be readily scored under the microscope as being colony-forming unit (CFU)-granulocyte, erythrocyte, monocyte, megakaryocyte (GEMM), CFU-granulocytes, monocyte (CFU-GM), or burst-forming unity-erythrocyte (BFU-E), and their relative frequency can be scored. Indeed in the absence of these supplements, colony formation was not observed. In normal spleen, CFU-GM are present in approximately equal ratios to BFU-E and at 10-fold excess over CFU-GEMM (ratios of 1.3:1.0:0.1, respectively), and CFU-GM represent approximately 0.01% of total spleen cells. By performing a ^{3}H-thymidine suicide killing assay, it was determined that approximately 3–4% of CFU-GM were in active cell cycle in the resting spleen. Following infection with *L. donovani*, total spleen hematopoietic activity demonstrably increased. CFU-GM increased almost twofold at day 7, fourfold at day 14, 22-fold at day 28, and 27-fold by day 56 of infection. Less marked changes occurred within the other progenitor populations. Similarly, the proportion of CFU-GM in active cell cycle had increased to over 50% at day 42 of infection. These changes were clearly dependent upon T-cell function rather than the presence of parasite *per se*, as no such alterations in hematopoietic activity were seen in the spleen of SCID mice, even though parasite burden was substantially higher than that of control mice. Examination of cytokine and chemokine mRNA accumulation by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) indicated that the major changes noted in control vs. SCID mice that were associated with this increase in hematopoietic activity were upregulation of the CSFs – GM-CSF, G-CSF, and M-CSF. These studies also indicated that the dramatic rise in CFU number occurred in parallel with the increase in parasite multiplication noted from day 14 to day 28 of infection, suggesting some link to these two events. Of importance, these changes were not localized to the spleen, and similar increases in hematopoietic activity were also observed in the infected bone marrow and in the blood. Analysis of blood was particularly informative. CFU-GM increased fivefold within 5 h of infection, suggesting rapid mobilization of progenitor cells into the blood stream from the bone marrow and the capacity of the blood to seed additional myeloid progenitor cells into the spleen (39).

We were able to demonstrate also that bone marrow stromal macrophages and stromal cell lines of macrophage origin could be infected with *L. donovani* and stimulate these cells to produce additional GM-CSF and TNFα (93). Strikingly, this effect of *L. donovani* infection was sufficient to allow the generation of CFU-GM in the absence of additional CSF supplementation of the media. In contrast, we could find no evidence that progenitor cells themselves were infected or in any way altered in function by *L. donovani* parasites.

Together, these data suggested that infection moderates the levels of CSF such that myeloid progenitor cell activity is enhanced and the number of myeloid cells increases proportionally. Such an increase in myeloid cell numbers is readily observed in the infected spleen. For one interpretation for how these changes might affect the outcome of infection, the 'safe target' hypothesis, initially proposed by Modabber and colleagues (94) to explain the link between GM-CSF production and susceptibility to *Leishmania major* infection, could be forwarded. However, differences in the parasite preference for immature (*L. major*) and mature (*L. donovani*) macrophages (95)

and the observation that GM-CSF is required for optimal resistance to *L. donovani* (96) questions whether this was a valid explanation of the data.

The local regulation of DC development

More recently, we have investigated another more subtle effect of this change in hematopoietic activity, which may more readily explain the correlation between increased hematopoietic activity and increased parasite growth and host susceptibility. Direct examination of DC subpopulations in the spleen showed that dramatic changes occurred over the same time frame as discussed above. Whereas in the resting spleen and at early times p.i. the majority of splenic CD11c^{hi} DC belong to one of the three main and extensively characterized subpopulations (CD4^{+}, CD8α^{+}, and double negative) (97, 98), by day 21 of infection, a substantial increase in CD11c^{lo} CD45RB^{+} DCs has occurred. In resting spleen, these represent less than 1% of the total spleen, whereas 28 days p.i. with *L. donovani*, DCs with this phenotype increase in number considerably. Cells with similar phenotype have been shown to respond to lipopolysaccharide (LPS) stimulation with the production of IL-10 and to be associated with the developmental regulatory IL-10-producing CD4^{+} T cells (99). CD11c^{lo}CD45RB^{+} DCs from *L. donovani*-infected mice also accumulate large amounts of IL-10 mRNA. Thus, infection appears to promote the development of DCs with regulatory potential. Adoptive transfer of these DCs into infected hosts markedly increases the parasite burden, measured 1 week later (Maroof *et al.*, unpublished data).

We were particularly interested, however, to understand the source of these or other types of DCs with regulatory function. One possibility, difficult to exclude experimentally in the intact mouse, is that they are recruited either from blood or following new production in the bone marrow. However, we wished to explore the possibility that these cells may be locally produced and reflect another facet of the increased hematopoietic activity we have observed in the spleen. We used an *in vitro* culture system employing c-kit^{+} lin^{-} bone marrow-derived progenitor cells from B6.CD45.1 mice and stromal cells isolated from naïve or *L. donovani*-infected B6 (CD45.2) mice. Crossing this allotypic barrier allowed unequivocal identification of DCs as being of progenitor origin. These cultures were not supplemented with any additional growth or differentiation factors. The spleen stromal cells used comprise a heterogeneous mixture of cell types, though they are predominantly ER-TR7^{+} fibroblasts and CD68^{+} macrophages with only minor contamination with endothelial cells.

We found that DCs could indeed be generated from progenitor cells under these conditions. Strikingly, phenotypic analysis revealed that essentially all DCs recovered were CD11c^{lo}CD45RB^{+} and had features of immature DCs (intracellular MHC class II, few dendrites, low expression of costimulatory molecules). These DCs could be matured using TNFα to become more dendritic in nature and to transport MHC class II to the plasma membrane. When functionally tested, these DCs had potent inhibitory effects on a mixed lymphocyte reaction driven by CD11c^{hi} DCs, and this effect could be largely reversed by IL-10R monoclonal antibody (mAb). Thus, stromal cells from the resting spleen appear largely to direct the development of regulatory DCs from progenitor cells. This finding may suggest a means of local production of such DCs (Svensson *et al.*, manuscript submitted). We then examined the ability of stromal cells from *L. donovani*-infected mice to support the development of DC. We found two major differences in the output of DCs from these cultures. First, the number of CD11c^{lo}CD45RB^{+} DCs with regulatory function increased approximately fivefold in cultures supported by stromal cells derived from infected mice compared to stromal cells from naïve mice. Second, on a per cell basis, DCs that developed on stromal cells from infected mice were more potent in inhibiting mixed lymphocyte reaction had greater accumulation of IL-10 mRNA and were less readily blocked in their function by anti-IL10R mAb than their counterparts that developed on stromal cells from naïve mice. Collectively, these data lead us to suggest that a major mechanism underlying immune dysregulation in the later stages of *L. donovani* infection is an alteration in the numerical balance as well as the functional properties of DC with regulatory vs. stimulatory capacity (Svensson *et al.*, manuscript submitted) (*Fig.* 3). Although we have not formally shown that such DCs induce *Leishmania*-specific regulatory T-cell populations during infection, it has been documented that such T cells exist in other *Leishmania* infections (100) as well as being present in mice with chronic VL (25, and Maroof, unpublished data). It remains to be determined by which mechanism stromal cells regulate DC development and how the function of stromal cells is altered during *L. donovani* infection. Analysis of stromal cells derived from various gene-targeted mice and the use of gene profiling should provide further clues.

In addition to the definition of a new regulatory pathway operating in *L. donovani*-infected mice, these studies suggest that the stromal cells have a general immunoregulatory role, determining indirectly through their influence on DC development the outcome of infection, autoimmune disease, and

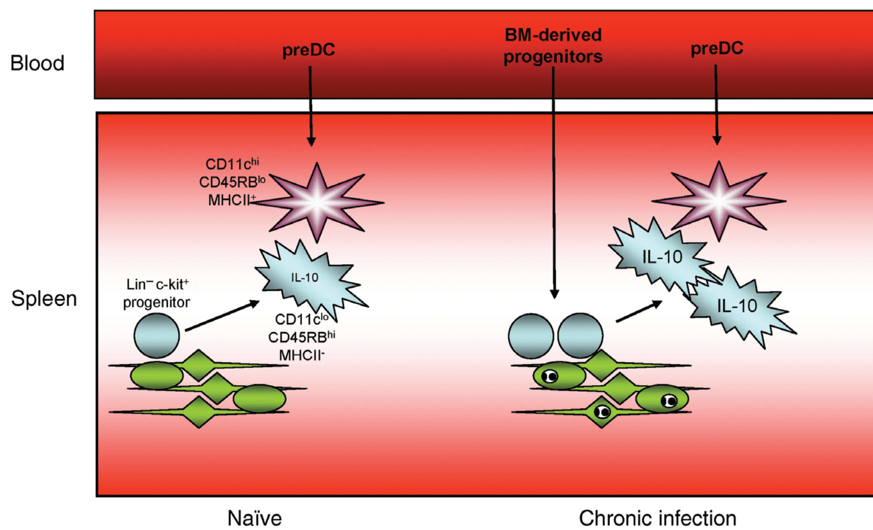

Fig. 3. Stromal cell regulation of DC subset balance in the spleen of *Leishmania donovani*-infected mice. Stromal cells have the capacity to support the differentiation of hematopoietic progenitor cells. In the resting murine spleen, stromal fibroblasts and macrophages induce the differentiation of $Lin^{-}c\text{-}kit^{+}$ cells into dendritic cells (DCs) with an immature phenotype and potent immunosuppressive activity. In resting mice, the numbers of such immature DCs are similar to that of conventional $CD11c^{hi}$ DCs. Following infection with *L. donovani*, bone marrow-derived progenitor cells are recruited, stromal cell function is enhanced, and the balance of DCs with stimulatory and suppressive properties is altered.

the host response to cancer. Stromal cells are diverse in their functions and may be tissue-specific in their composition. As we have shown (see above), remodeling of stromal cells readily occurs during chronic inflammation, and stromal cell changes have been noted in many disease states. The concept that stromal cells might differentially regulate local DC development under different circumstances is of particular future importance for understanding immune dysfunction in a range of parasitic infections.

Splenomegaly and the regulation of T-cell memory

Splenomegaly is such a dramatic feature of VL that we have continued to assess the possible contributions of this syndrome to immune responsiveness during VL. A number of observations suggested that examination of the memory T-cell response might also be informative. First, one of the key features of human VL is an increase in susceptibility to opportunist infections, including many to which prior exposure may have been likely (1). Second, clinical studies have indicated that the peripheral pool of $CD45RO^{+}$ T cells is depleted in acute VL and rises only with successful therapy (101). It is unclear whether this observation is due to sequestration of $CD45RO^{+}$ cells in systemic sites or to subsequent replacement by T cells of new specificity. Third, surprisingly little is known about the development of memory T cells during experimental VL. Important questions remain about how memory T-cell responses are generated, the subsets of memory T cells that predominate, and their respective capacities to mediate long-term protection. Finally, one of the central models underlying studies of T-cell memory is that the compartment size is fixed, allowing competitive interactions between T cells for survival within the memory pool (102, 103) (Fig. 4). Such models have not been tested under conditions of chronic parasitic infection. In the following sections, we review recent data on these issues.

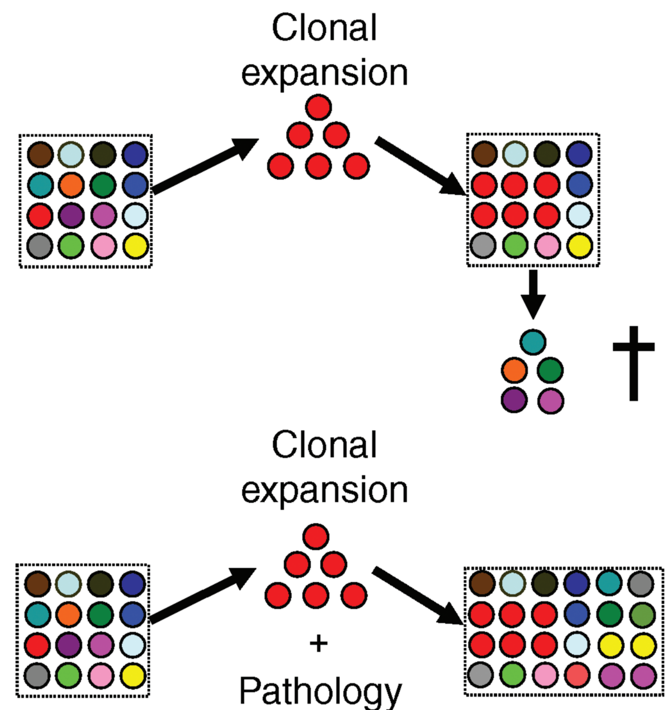

Fig. 4. Splenomegaly and the size of the 'memory' compartment. Existing data suggest that limitations in the size of the memory cell compartment result in competition for space among memory T cells. Thus, new memory cells generated following clonal expansion will cause loss or attrition of T cells with other specificities from the memory pool (top cartoon). Where chronic splenomegaly occurs, recent data suggest that the functional size of the memory compartment is increased to accommodate memory cells specific to the causative infection as well as increased numbers of heterologous and pre-existing antigen-specific memory cells generated as a result of bystander proliferation (bottom cartoon).

Development of CD4^{+} memory T cells after *L. donovani* infection

CD4^{+} T-cell activation can be observed within 18 h of infection, but surprisingly there have been few studies that have phenotyped the longer term changes in CD4^{+} T cells over the course of *L. donovani* infection. We therefore studied the progression of the CD4^{+} T-cell response using a panel of cell surface antigens used to define memory T-cell populations. In BALB/c mice, the number of splenic CD4^{+} T cells increases during *L. donovani* infection from around 13×10^6 to 45×10^6 over a 56-day period. Bromo-2′-deoxyuridine labeling indicated that approximately 14% of these cells underwent division during a 7-day pulse period, a turnover rate twice that of naïve T cells. Analysis of these CD4^{+} T cells using conventional markers to distinguish memory T cells (CD62L and CD44), indicated that as many as one-third of memory cells in infected mice had undergone division in a 7-day period. Thus, *L. donovani* appears to favor cell division and ultimate expansion of memory CD4^{+} T cells as measured at the population level.

Competition in the CD4 memory compartment

The expansion of CD4^{+} T cells as a consequence of infection raises the issue of how many of these were actually specific to *L. donovani* antigens and whether infection also promoted 'bystander' proliferation of cells of irrelevant antigen specificity. Bystander proliferation of T cells is a well-documented response to exposure to cytokines, such as IL-2 or IL-15, and is independent of T-cell receptor (TCR) signaling (104, 105). To address this issue, we have studied first the impact of *L. donovani* infection on a population of CD4^{+} T cells specific for chicken ovalbumin (OVA). *In vitro* recall responses using antigen- presenting cells (APCs) loaded with a variety of forms of leishmanial antigen in combination with polyclonal OVA-specific T cells, OVA-specific DO.11 T-cell hybridomas, or DO.11 TCR transgenic naïve and effector T cells all fail to detect any cross-reactivity between *L. donovani* and OVA, making this a suitable specificity for addressing the impact of infection on a heterologous T-cell population. Two distinct protocols were employed. In the first, a classical adoptive transfer approach was used, which allows determination of the fate of OVA-specific memory T cells in the absence of antigen. TCR transgenic DO.11 T cells were stimulated *in vitro*, expanded with IL-2, separated from any residual contaminating APCs, and then adoptively transferred into antigen-free BALB/c recipients. T cells were rested in these recipients for 2–4 weeks before the mice were infected with *L. donovani*. The fate of these DO.11 memory cells was then followed over 56 days of infection. As expected, in the absence of antigen, DO.11 T cells are lost over time from the spleen of naïve mice. Strikingly, the rate of loss of DO.11 memory T cells was significantly more rapid when mice were infected with *L. donovani*, and DO.11 cells in these infected mice were almost undetectable by day 56 p.i. These data indicate that, as might be predicted by models where the size of the memory compartment is finite, new expansion of CD4^{+} memory cells (including some presumably with specificity for *L. donovani*) can cause attrition of a defined pool of pre-existing memory cells (Polley *et al.*, manuscript submitted).

While informative, the experiment described above does not reflect what is perhaps the more common scenario, i.e. one where antigen in the form of immune complexes or as viable persistent pathogens is present in the host for long periods of time. Hence, of more biological significance is the effect that *L. donovani* infection might have on the fate of pre-existing memory cells for which antigen is still available, albeit at limiting concentrations. We therefore adoptively transferred naïve DO.11 T cells into mice, immunized them with OVA in complete Freund's adjuvant (or Ribi adjuvant) and then left them for 4 weeks, prior to infection with *L. donovani*. Control experiments indicated that 4 weeks after immunization, residual antigen was able to minimally stimulate the division of newly transferred activated DO.11 T cells. In stark contrast to the data obtained in an antigen-free transfer environment, *L. donovani* infection in this setting caused significant expansion of the DO.11 memory T-cell population (Polley *et al.*, manuscript submitted). These data suggested that for CD4^{+} T cells under these experimental conditions (i) the available pool size for memory CD4^{+} T cells may not be limiting and (ii) that cognate antigen is required in order to facilitate expansion of DO.11 cells following *L. donovani* infection. What remains to be resolved is how antigen operates in this system. One explanation is that antigen is needed for the long-term survival of DO.11 cells, such that they are then able to respond to bystander cytokine signals generated later in infection. Alternatively, the activation of the DO.11 memory response might be facilitated by heightened APC activity in *L. donovani*-infected mice, allowing for an enhanced presentation of residual OVA. Given the changes associated with the lymphoid environments discussed above, which mainly serve to limit the host's capacity for T-cell activation, the latter alternative may appear unlikely.

Competition in the memory CD8^{+} T-cell compartment and heterologous protection

Arguably, the generation and regulation of memory CD8^{+} T cells is better defined than for memory CD4^{+} T cells. We

therefore also examined how memory $CD8^+$ T cells specific for a heterologous antigen (listeriolysin of *Listeria monocytogenes*) behave when faced with *L. donovani* infection. As with $CD4^+$ T cells, the number of memory $CD44^{hi}$ $CD8^+$ T cells in mice infected with *L. donovani* increases (approximately 10-fold over a 56-day period of infection). In normal mice infected with *L. donovani*, there is no expansion of $CD8^+$ T cells recognized by an $LLO_{91-99}/H2K^d$ tetramer, suggesting that no T cells activated in normal mice as a consequence of *L. donovani* infection are able to recognize this listeriolysin epitope. In contrast, when mice were vaccinated with a low dose of viable *Listeria*, rested for 6 weeks to allow memory cell formation, and then subsequently infected with *L. donovani*, a marked expansion of $tetramer^+$ $CD8^+$ T cells was observed. In order to examine the functional consequences of this expansion for host protection against *Listeria* infection, we challenged mice with a potentially lethal dose of *Listeria*. Immunization with low doses of *Listeria* alone induced only low levels of protection against challenge (approximately 1–2 logs reduction in spleen CFU). A similar level of protection was obtained when *Listeria*-naïve mice were infected with *L. donovani* (probably as a result of local macrophage activation). More striking, however, when *Listeria*-immune mice were infected with *L. donovani*, protection against subsequent *Listeria* challenge was almost complete (five to six log reduction in spleen CFU). Hence, the bystander proliferation of listeriolysin-specific $CD8^+$ T cells appears to have a significant effect on protection to reinfection. $CD8^+$ $tetramer^+$ cells stimulated to expand in the highly inflammatory environment created by *L. donovani* infection also had heightened capacity to produce IFNγ, as measured at the single cell level. These data indicate that under these controlled conditions, the impact of *L. donovani*-induced immune dysfunction and splenomegaly appears to be paradoxically beneficial to the host in terms of resistance to this previously encountered heterologous infection (Polley *et al.*, manuscript submitted).

Why in at least two settings ($CD4^+$ T cells in the presence of limiting antigen and $CD8^+$ T cells) do we not see evidence of attrition of pre-existing memory T cells in the face of subsequent infection with *L. donovani*? One simple possibility is that there is insufficient expansion or excess loss of *L. donovani*-specific T cells to cause this effect, e.g. compared to the extensive clonal expansion that accompanies viral infection (102). However, the observation that attrition is observed under the strict conditions of an antigen-free environment suggests that this possibility might not be the case. An alternative, perhaps intuitive explanation is that the 'size' of the memory compartment may well bear some relationship to the physical size of the lymphoid compartment itself. The cardinal feature of *L. donovani* infection, namely splenomegaly, may therefore reduce competitiveness in favor of coexistence within a larger compartment (*Fig.* 4). Further studies are clearly required to formally distinguish these possibilities.

Outlook

High-dose models of VL may soon be replaced by the advent of low-dose models, which more closely mimic the natural route of infection (106), and only future experimentation will determine whether major differences in disease outcome, host genetic control, and/or the cellular and molecular basis for resistance and susceptibility in different tissue sites are apparent. Nevertheless, the use of high-dose models for the identification and characterization of microarchitectual changes associated with disease progression in mice, and the changes to macrophage, DC, and lymphocyte populations observed will continue to allow us to devise strategies to modulate immunity to promote disease resolution without pathology and to maintain long-term immunity. Perhaps one of the greatest challenges in the near future will be to make these strategies deliverable in a form and at a cost that is realistic for large-scale implementation into some of the poorest nations on earth.

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