A Role for Tumor Necrosis Factor- α in Remodeling the Splenic Marginal Zone during *Leishmania donovani* Infection

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The development of secondary lymphoid organs is a highly regulated process, mediated by tumor necrosis factor (TNF) family cytokines. In contrast, the mechanisms controlling changes in lymphoid architecture that occur during infectious disease are poorly understood. Here we demonstrate that during infection with *Leishmania donovani*, the marginal zone of mice undergoes extensive remodeling, similar in extent to developmental abnormalities in mice lacking some TNF family cytokines. This process is selective, comprising a dramatic and rapid loss of marginal zone macrophages (MZMs). As a functional consequence, lymphocyte traffic into the white pulp is impaired during chronic leishmaniasis. Significantly, MZMs were preserved in *L. donovani*-infected B6.TNF- $\alpha^{-/-}$ mice or mice that received anti-TNF- α antibodies, whereas studies in CD8⁺ T-cell-deficient mice and in mice lacking functional CD95L, excluded a direct role for either cytotoxic T lymphocyte activity or CD95-mediated apoptosis in this process. Loss of MZMs was independent of parasite burden, yet could be partially prevented by chemotherapy, which in turn reduced endogenous TNF- α levels. This is the first report of an infectious agent causing selective and long-lasting changes to the marginal zone via TNF- α -mediated mechanisms, and illustrates that those cytokines involved in establishing lymphoid architecture during development, may also play a role in infection-induced lymphoid tissue remodeling. (Am J Pathol 2002; 161:429–437)

The splenic marginal zone forms the boundary between the red pulp and specialized areas of lymphocyte accumulation in the white pulp.¹ Within the network of reticular cells comprising the marginal zone,² two novel macro-

phage populations are found, as well as B cells, dendritic cells, and trafficking lymphocytes. Marginal metallophilic macrophages (MMs) are located at the inner border of the marginal zone, adjacent to the white pulp, whereas marginal zone macrophages (MZMs) are present at the outer boundary of the marginal zone, adjacent to the red pulp.¹ The marginal zone has diverse functions, including the direction of lymphocyte traffic into the white pulp,³ and the phagocytosis of blood borne pathogens.¹ In addition, it acts as the site for the generation of type II T-independent humoral responses,⁴ reflecting both the presence of unique marginal zone B cells^{5,6} and the specialization of MZMs for the uptake of T-independent antigens.⁷

During ontogeny, development of the splenic marginal zone is under complex control, mediated by members of the tumor necrosis factor (TNF) family of cytokines and their downstream signaling molecules.^{8–13} Studies in gene-targeted mice have begun to identify differences in the factors necessary for development of the various cellular components of the marginal zone. Thus, in the absence of lymphotoxin- α ^{14–16} or the transcription factor relB,¹¹ the marginal zone is completely absent. In contrast, TNF- α -deficient mice show defects in the marginal sinus, but retain both MMs and MZMs, albeit with some disruption to their positioning.^{17,18} Likewise, whereas nuclear factor- κ B1-deficient mice have fully intact marginal zones,¹¹ mice deficient in nuclear factor- κ B2 retain MZMs but lose both MMs and the marginal sinus.^{9,12} These studies suggest that organization of the marginal zone is achieved through a combination of independent and overlapping mechanisms. Once established, maintenance of the marginal zone appears to fall mainly to lymphotoxin- α , as blockade of this cytokine, but not TNF- α has a dramatic impact on marginal zone structure.^{16,19}

In contrast to the well-organized splenic architecture seen in naive animals, tissue disruption has been frequently noted during infectious and other chronic inflam-

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matory diseases.^{20–24} However, there is little information on the underlying mechanism of tissue remodeling during these pathological states, although it is known that aberrant regulation of TNF family members often accompanies such conditions.^{20,23,25}

The protozoan parasite *Leishmania donovani* causes visceral leishmaniasis (VL), a disease that remains a significant clinical problem in many parts of the world.²⁶ In genetically susceptible mice, the spleen and bone marrow are sites of persistent infection^{27,28} and the spleen shows many pathological features associated with human VL, including germinal center involution and loss of follicular dendritic cells.^{20,28} Here we report that *L. donovani* infection also induces dramatic remodeling of the splenic marginal zone. Most notably, infection induces a rapid and selective loss of MZMs, whereas disruption to the MMMs and marginal sinus is less pronounced. We have used a number of different experimental approaches to implicate TNF- α as the key mediator of this remodeling process, showing that cytokines involved in establishment of lymphoid architecture during development may also play an active, but distinct role in its breakdown.

Materials and Methods

Animals and Parasites

BALB/c and C57BL/6 mice were purchased from Tuck and Co. (Battlesbridge, UK) and were housed under conventional conditions. Mice deficient in TNF- α (B6.TNF- $\alpha^{-/-}$),¹⁰ CD95L (B6.gld),²⁹ and $\beta 2m$ (B6. $\beta 2m$)³⁰ were obtained from Bantin and Kingman (Hull, UK) or the Jackson Laboratories (Bar Harbor, ME) and bred at the London School of Hygiene and Tropical Medicine under barrier conditions. *L. donovani* (LV9) was maintained by passage in Syrian hamsters and amastigotes were isolated from infected spleens, as previously described.²⁸ Mice were infected at 6 to 8 weeks of age by injecting 2×10^7 amastigotes intravenously via the lateral tail vein. Mice were sacrificed at times indicated in the text by cervical dislocation and bled by severing the aorta. Livers and spleens were removed and parasite burden was determined from Giemsa-stained impression smears.³¹ Parasite burden was expressed in Leishman-Donovan units (LDUs), in which LDU is the number of amastigotes per 1000 host nuclei, multiplied by the organ weight.²⁸

Chemotherapy and Monoclonal Antibody (mAb) Treatment

Mice were treated with a leishmanicidal dose of sodium stibogluconate, a pentavalent antimonial (Pentostam, 31.3% (w/w) Sb^v; Wellcome, Beckenham, UK) 14 days after infection. Mice received daily subcutaneous injections of 400 mg/kg Sb^v in 200 μ l of 0.25% (w/v) methylcellulose for 5 days, based on established dosing regimes.³² Blockade of TNF- α was achieved by administration of 0.3 mg of hamster anti-mouse TNF- α mAb (TN3-19.12;³³) on days 14,

19, and 24 after infection. Control mice received the same amount of normal hamster IgG (ICN, Thame, UK).

Immunohistochemistry

Antibodies used for histology included anti-MAdCAM-1 (MECA-367), anti-CD169 (3D6.112), anti-metallophilic macrophages (MOMA-1) (all from Serotec, Kidlington, UK), and anti-MZMs (ER-TR9; a gift from G. Kraal, Free University, Amsterdam, Holland) antibodies. Tissue staining of spleens was conducted on 6- μ m acetone-fixed sections stained with the above primary antibodies and appropriate secondary detection reagents according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK), and as previously described.²⁸ In some experiments, mice were injected intravenously with 200 μ l of 5% [v/v in 0.9% (w/v) NaCl] carbon particles (India ink; Rowney and Co., Bracknell, UK) to allow visualization of MZ macrophages in the spleen, as previously described.³⁴ Sections were dehydrated and mounted before microscopic examination. In some cases, sections were counterstained with hematoxylin (Sigma, Poole, UK) before dehydration and mounting.

Lymphocyte Trafficking Experiments

Lymphocytes were isolated from naïve splenocytes using Histopaque 1083, according to the manufacturer's instructions (Sigma). Cells were labeled with Hoechst 33342 (6 μ g/ml; Boehringer, Mannheim, Germany) at 1×10^7 cells per ml in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% (v/v) fetal calf serum (Sigma) at 37°C for 15 minutes. Cells were washed twice and then incubated at room temperature for 30 minutes, before washing twice more. Mice were administered 1×10^7 Hoechst 33342-labeled cells in 200 μ l of RPMI 1640 via the lateral tail vein. After 2 hours, mice were sacrificed, and spleens were removed and embedded in OCT compound (Raymond Lamb, London, UK) on cork blocks before snap-freezing in isopentane/liquid nitrogen. The distribution of Hoechst 33342-labeled cells was analyzed in 20- μ m, acetone-fixed sections using a fluorescent microscope under UV illumination.

ELISPOT Analysis

The frequency of splenocytes producing TNF- α was determined by direct *ex vivo* ELISPOT assay, as described in detail elsewhere,³¹ using mAb TN3-19.12 as capture antibody and a polyclonal anti-murine TNF- α as detecting antibody.³⁵

Results

MZMs Are Lost during *L. donovani* Infection

In genetically susceptible C57BL/6 mice, parasite number in the spleen increases slowly throughout the first 56 days of infection and then numbers stabilize or marginally

Figure 1. The course of *L. donovani* infection in the spleen of BALB/c (●) and C57BL/6 (▲) mice. Half of the infected BALB/c (○) and C57BL/6 (△) mice received Sb^v (400 mg/kg s.c.) at day 14 after infection for 5 days. **A:** Parasite burdens measured throughout 180 days of infection. **B:** Spleen weights in mice measured throughout the same time period. Data represent the mean $\pm$ SE ($n = 3$ to 4 animals per group at each time point). Data are from one representative experiment of four performed.

decline (Figure 1A). Splenomegaly is a feature of infection, with the spleen reaching up to 3 to 5% of body weight at the peak of infection (Figure 1B). To follow the fate of MZMs during infection, two approaches were taken, using both the MZM-specific mAb ER-TR9,³⁶ and the specialization of these cells for carbon uptake.^{1,34} In naive mice, ER-TR9⁺ MZMs were localized in the marginal zone, forming a continuous ring around the white pulp (day 0 after infection, Figure 2A), confirming the specificity of this antibody as described previously.³⁶ However, this distinct pattern of staining was rapidly disrupted after infection (day 14 after infection, Figure 2A) and ER-TR9⁺ MZMs were markedly reduced in number at all subsequent times (days 28 to 180 after infection, Figure 2A). Infection of BALB/c mice with *L. donovani* similarly gives rise to a chronic infection in the spleen, although both parasite burden and splenomegaly are greater than that seen in C57BL/6 mice (Figure 1). Loss of ER-TR9⁺ MZMs was indistinguishable between BALB/c and C57BL/6 mice, (data not shown), suggesting that

Figure 2. *L. donovani* infection leads to the loss of MZ macrophages in the spleens of C57BL/6 mice. **A:** The mAb ER-TR9 was used to detect MZ macrophages in naive (day 0 after infection) and infected mice at the times indicated. **B:** Uptake of carbon particles by MZ macrophages. C57BL/6 mice received India ink (intravenously) either 1 day before sacrifice or 29 days before being sacrificed, as indicated above the panels. The distribution of carbon particles in the spleen was analyzed in 20 μ m of tissue sections from naive mice (day 0 after infection) and from mice at day 28 after infection. Half of the infected mice received chemotherapy (+Sb^v), as indicated. Data are representative of one of three experiments performed. Original magnifications, $\times 100$.

parasite burden per se is not the only factor that determines the development of pathology.

Changes in the number of ER-TR9⁺ MZMs could potentially result from modulation of ER-TR9 expression un-

der inflammatory conditions, rather than actual loss of MZMs. To address this issue, we took advantage of the known specificity of MZMs for uptake of carbon particles.³⁴ To confirm the specificity of this approach and its ability to provide a long-term marker for MZMs, naive mice were examined at various times after India ink injection. Carbon particles were restricted to MZMs when examined 1 day later (India ink, 1 day; Figure 2B), and MZMs were still readily observed in the marginal zone 29 days later (India ink, 29 days; Figure 2B), although a few cells containing carbon could be observed in the red pulp at this time (Figure 2B). When mice were administered India ink before infection with *L. donovani* and then examined 28 days later, few cells containing carbon particles could be detected, either in the marginal zone or in the red pulp (India ink, 29 days; Figure 2B). Furthermore, we observed little uptake of freshly injected carbon particles in spleens at day 28 after infection (India ink, 1 day; Figure 2B), indicating that those MZMs that were lost throughout the preceding 28 days had not been replenished, either in the marginal zone or elsewhere, by cells with similar phagocytic capacity. Thus, by these two independent methods, we conclude that MZMs are significantly depleted from the marginal zone during *L. donovani* infection.

Changes in Other Marginal Zone Cell Populations during *L. donovani* Infection

We next examined the fate of the other major population of macrophages found in the marginal zone, the MMMs. We examined expression of two molecules (MOMA-1 and CD169) that have been previously been shown to be co-expressed by MMMs in naive mice.^{37,38} Although CD169 is weakly expressed on MZMs and red pulp macrophages,³⁸ distribution of strongly CD169-positive cells in this study is likely to represent that of MMMs. In naive mice (day 0 after infection), expression of MOMA-1 and CD169 were clearly observed (Figure 3), and staining of spleens from mice previously injected with India ink clearly demonstrated both the nonoverlapping distribution of these markers with carbon particles in the MZMs, and the localization of MMMs to the inner face of the marginal zone (data not shown). Similarly, CD169 staining formed a continuous ring around the white pulp in the spleen of naive mice (Figure 3), with localization on the inner side of the marginal zone. At day 14 after infection, we found a dramatic decrease in expression of MOMA-1 after *L. donovani* infection (Figure 3). In contrast, expression of CD169 was minimally affected throughout the first 28 days of infection (Figure 3). These data suggest that inhibition of MOMA-1 expression may occur as a consequence of inflammation. It is unlikely that infection with *L. donovani* directly affected expression of MOMA-1, as MOMA-1⁺ MMMs infected with *L. donovani* can be clearly identified early during infection.³⁹ Nevertheless, the persistent expression of CD169 throughout the first 28 days after infection indicates that MMMs are substantially retained at a period when MZMs have been essentially depleted from the spleen. MMMs subsequently become

Figure 3. *L. donovani* infection alters the expression of other non-MZ macrophage markers in the spleens of C57BL/6 mice. The expression of MOMA-1 and CD169 (associated with MM macrophages), as well as MAdCAM-1 (found on splenic endothelial cells), was analyzed in naive and infected mice at the times indicated. Half of the infected mice received chemotherapy (+SbV), as indicated. Data are representative of one of three experiments performed. Original magnifications, $\times 100$.

less abundant at the peak of splenomegaly and thereafter, and have a somewhat more patchy distribution (day 56 and 180, Figure 3A).

The endothelial cells of the marginal sinus are characterized by expression of the addressin MAdCAM-1.⁴⁰ In naive mice, MAdCAM-1 staining was localized to the marginal zone and formed a continuous ring around white pulp regions, often separating MZMs and MMMs (Figure 3). Expression of MAdCAM-1 followed a similar course to that of CD169 up to day 28 after infection, with minimal disruption to the integrity of the marginal sinus at these early times. Although some further disruption occurred by day 56 after infection this appeared transient and the marginal sinus was partially restored by day 180 after infection (Figure 3). Similar changes in MOMA-1, CD169, and MAdCAM-1 expression were also observed in BALB/c mice infected with *L. donovani* (data not shown). Together, these data indicate that the impact of infection on the marginal zone is selective at the early stages of infection, targeting mainly MZMs, whereas MMMs and the marginal sinus are only significantly affected at later times.

Figure 4. *L. donovani* infection profoundly alters the retention and migration of lymphocytes within the spleen of C57BL/6 mice. Fluorescent Hoechst 33342-labeled splenic lymphocytes were administered (intravenously) into naive mice and mice at day 28 after infection, as indicated. Spleens were removed 2 hours later, fixed, mounted, and then photographed under UV illumination (20 μ m tissue sections). Half of the infected mice received chemotherapy (+Sb^V), as indicated. Data are representative of one of three experiments performed. Original magnifications, $\times 100$.

The Loss of MZMs during L. donovani Infection Disrupts Lymphocyte Migration

MZMs have a key role in directing lymphocyte migration.³ Therefore, we examined the consequences of the selective loss of these cells on local lymphocyte trafficking into the white pulp of the spleen. Lymphocytes were labeled with Hoechst 33342 and then injected intravenously into naive and *L. donovani*-infected C57BL/6 mice. The distribution of these lymphocytes was then monitored 2 hours later by fluorescent microscopy (Figure 4). In naive mice, donor lymphocytes were seen to interact with MZMs, with some lymphocytes already having migrated into the white pulp (Figure 4,³). In stark contrast, very few fluorescent lymphocytes were found in spleens of infected mice at 28 days after infection (Figure 4). In addition, no defined pattern of cellular migration was observed, although some Hoechst 33342-labeled cells were occasionally seen interacting with the few remaining MZMs (indicated by carbon labeling). The reduced number of fluorescent cells in the spleens of infected mice could not be attributed to splenomegaly at this stage of infection. Although a fourfold to fivefold increase in spleen size was ob-

served at 28 days after infection (Figure 1B), the number of fluorescent cells had decreased by greater than 10-fold in the field of view of each tissue section (Figure 4, data not shown). In contrast, we have observed no difference in Hoechst 33342-labeled lymphocyte migration into the liver and inguinal lymph nodes at the time point studied, regardless of infection (data not shown). However, other tissue sites have not been investigated.

MZMs Are Lost via a TNF- α -Dependent Mechanism

The mechanism underlying changes in lymphoid architecture during infection have only previously been addressed in a model of lymphocytic choriomeningitis virus (LCMV) infection. LCMV infects numerous cell types, including MZMs and targets the activity of cytotoxic CD8⁺ T cells.⁴¹ To determine whether the activity of cytotoxic T cells might also be involved in the loss of MZMs during *L. donovani* infection, we studied the distribution of MZMs and MMMs in *L. donovani*-infected $\beta 2$ M-deficient mice. The changes in marginal zone structure observed in these mice were identical to those seen in wild-type controls (data not shown), indicating that CD8⁺ T cells do not make a substantial contribution to marginal zone remodeling. Similarly, studies in CD95-deficient mice (B6.gld) suggest no role for apoptosis mediated via CD95-CD95L interaction in the loss of MZMs during this infection (data not shown).

TNF family members are essential for the proper development of the marginal zone. Whereas lymphotoxin- $\alpha^{-/-}$ mice fail to develop marginal zones,¹⁴⁻¹⁶ thus precluding them from this study, TNF- $\alpha^{-/-}$ mice have readily identifiable MMZ, MMMs, and a marginal sinus.¹⁸ Therefore, as we had previously shown that TNF- α is dramatically up-regulated during *L. donovani* infection,^{31,42} we next sought to determine whether overexpression of this cytokine may play a role in marginal zone remodeling. Staining of naive B6.TNF- $\alpha^{-/-}$ mice indicated that ER-TR9⁺ MZMs were present, although as previously reported their number was slightly reduced and their distribution somewhat more patchy than in the marginal zone of normal mice.¹⁸ The presence of MZMs in naive mice and their distribution was also confirmed by injection of carbon particles (Figure 5). Despite these minor changes, marginal zone function was intact, in as much as wild-type lymphocytes adoptively transferred into B6.TNF- $\alpha^{-/-}$ mice could readily migrate to the white pulp (Figure 5). We then infected the B6.TNF- $\alpha^{-/-}$ mice and examined marginal zone structure at day 28 after infection, the time at which selective loss of MZMs was most dramatic (Figure 2A), and a time at which C57BL/6 and B6.TNF- $\alpha^{-/-}$ mice have similar parasite burdens in the spleen (59 ± 9 LDUs versus 70 ± 20 LDUs, respectively). Staining of splenic sections with ER-TR9 antibody and studies on the uptake of carbon particles indicated that MZMs were still readily detectable in *L. donovani*-infected B6.TNF- $\alpha^{-/-}$ mice, compared to C57BL/6 mice infected for the same time (Figure 5, B and C). Equally important, the migration of lymphocytes into the white pulp was also

Figure 5. TNF- α mediates the loss of MZ macrophages in the spleen of *L. donovani*-infected mice. Fluorescent Hoechst 33342-labeled splenic lymphocytes were administered (intravenously) into naïve mice and in C57BL/6 and B6.TNF- $\alpha^{-/-}$ mice at day 28 after infection, as indicated (**A**). Mice also received India ink (intravenously) 1 day before being sacrificed (**B**). The mAbs ER-TR9 (**C**) and MOMA-1 (**D**) were used for detection of MZ and MM macrophages, respectively, as indicated. Mice also received India ink (intravenously) 1 day before being sacrificed. Note that panels in **A** correspond to panels in **B**. Data are representative of one of two experiments performed. Original magnifications, $\times 100$.

substantially preserved in *L. donovani*-infected B6.TNF- $\alpha^{-/-}$ mice (Figure 5A). Interestingly, MOMA-1 reactivity on MMMs was also partially preserved (Figure 5D), suggesting that MOMA-1 expression may be negatively regulated by TNF- α .

To confirm a role for TNF- α in regulating changes to the marginal zone during *L. donovani* infection, we also evaluated the impact of administering a neutralizing anti-TNF- α mAb to normal mice. Anti-TNF- α mAb (TN3-19.12) was administered between days 14 and 28 after infection, according to schedules previously determined to neutralize endogenous TNF- α .³³ This treatment was also found to inhibit the loss of MZMs, as judged by ER-TR9 staining (data not shown).

The Impact of Chemotherapy on Marginal Zone Remodeling

Parasite load in the tissue of mice can be dramatically reduced by the administration of antimonial chemotherapy (Figure 1A). Splenomegaly persists, albeit at a reduced level in drug-treated mice (Figure 1B). This provided a convenient model to more directly assess the impact of parasite burden on marginal zone remodeling. After chemotherapy, the number and distribution of MZMs, as determined by carbon uptake, was preserved compared to nontreated infected mice, and approached that seen in control uninfected mice (Figure 2B). Using ER-TR9 as a marker of MZMs, preservation of MZMs by chemotherapy was less pronounced, suggesting that expression of this molecule may also be subject to negative regulation during inflammation. Consistent with the retention of MZMs seen by carbon uptake, mice treated with chemotherapy also substantially retained the ability to direct the traffic of lymphocytes into the white pulp (Figure 4). Therefore, whereas drug treatment does not fully retain MZMs at numbers found in naïve mice, phagocytosis in the marginal zone and the mediation of lymphocyte migration are both functionally preserved.

Chemotherapy also had effects on both the MMMs and on cells of the marginal sinus. In drug-treated mice, CD169⁺ MMMs were substantially preserved throughout subsequent infection, although limited change in MOMA-1 expression occurred. Likewise, mice treated with chemotherapy showed little alteration in MAdCAM-1 expression (Figure 3).

Given that our data in B6.TNF- $\alpha^{-/-}$ mice and in mice treated with anti-TNF- α antibodies suggested a causal role for TNF- α in the loss of MZMs, we asked whether chemotherapy might act to protect MZMs by changing the levels of endogenous TNF- α being produced. ELISPOT assay demonstrated that *L. donovani*-infected mice have a dramatic increase in the frequency of TNF- α -producing cells in their spleens, compared to uninfected control mice (2250 ± 94 versus 32 ± 2 spot forming cells/ 10^6 spleen cells, in infected and control mice, respectively). This confirmed previous analysis of TNF- α expression in the spleen by both immunohistochemistry³¹ and by reverse transcriptase-polymerase chain reaction.⁴² Chemotherapy had a dramatic impact

on local TNF- α production, reducing the frequency of TNF- α -producing cells to levels seen in naïve mice (17 ± 20 spot forming cells/ 10^6 spleen cells).

Discussion

The efficiency of the mammalian immune system is in part attributable to the development of organized lymphoid tissue, in which lymphocyte responses are highly compartmentalized. As a result, both lymphoid organogenesis and the development of lymphoid tissue architecture are under precise control.^{8–12,14,15,17–19} We demonstrate here that during chronic infection, remodeling of the splenic marginal zone occurs, with dramatic consequences for lymphocyte traffic. Further, our data indicate that TNF- α , a key cytokine in orchestrating the normal lymphoid environment, can also play a role in its disruption under conditions of chronic inflammation.

Previous studies with viscerotropic strains of LCMV have noted destruction of MZMs, as well as red pulp macrophages and follicular dendritic cells. During this infection, cell death results from the direct action of CD8⁺ cytotoxic T cells that target LCMV-infected cells.⁴¹ This host-mediated pathology resulted in severe immunosuppression caused by impaired antigen presentation to lymphocytes.⁴³ Significantly, TNF- α was not crucially involved in MZM destruction after LCMV infection.⁴¹ Conversely, loss of MZMs during infection with *L. donovani* occurs in B6. $\beta 2m^{-/-}$ mice, excluding a role for CD8⁺ cytotoxic T cells, and our data indicates that TNF- α plays a major role in this event.

Three key aspects of our data implicate TNF- α as a mediator of marginal zone remodeling. First, TNF- α is overexpressed during chronic *L. donovani* infection. Analysis by ELISPOT assay indicates that as many as 1 in 450 spleen cells directly make TNF- α when isolated from infected mice, compared to <1 in 30,000 that do so in naïve mice. The high frequency of TNF- α -producing cells is consistent with the very high levels of this cytokine previously noted by immunocytochemistry.³¹ TNF- α production is first noted by day 3 after infection within the marginal zone, although MZMs and MMMs are infected within hours of intravenous inoculation of *L. donovani*.³⁹ Infection of various macrophage populations with *Leishmania in vitro* suggest this parasite is a poor inducer of TNF- α ,⁴⁴ and the delay in kinetics for TNF- α production *in vivo* is likely to represent a requirement for early interferon- γ production and subsequent macrophage priming for TNF- α production.^{35,39} At later stages of infection, notably when the marginal zone is being remodeled, the distribution of TNF- α -producing cells is widespread, and likely has many diverse cellular origins.³¹

Second, interventions that reduce TNF- α production or its activity are able to limit the loss of MZMs. Both neutralization of TNF- α and reducing endogenous TNF- α by chemotherapy protect against MZM loss. In the case of chemotherapy, TNF- α is reduced to the levels seen in naïve mice. However, it should be noted that chemotherapy also leads to reduced expression of other proinflammatory cytokines (CR Engwerda, unpublished results).

Recent data suggest that pentavalent antimony may have important effects on signaling cascades, by inhibiting SHP-1 activity,⁴⁵ although it remains to be determined if this is the basis of changes in the profile of proinflammatory cytokines after treatment. Third, loss of MZMs is much less evident in mice that lack TNF- α . Although B6.TNF- $\alpha^{-/-}$ mice have some alterations in their marginal zone structure, the presence of MZMs and MMMs makes them readily traceable during the course of infection. Importantly, although TNF- $\alpha^{-/-}$ mice and wild-type mice display a different course of infection in the liver,⁴⁶ the course of infection and absolute parasite burden is similar in the spleen of infected mice. This rules out differences in parasite load as a cause of the protection of structure observed.

Studies in a variety of gene-targeted mice have illustrated the complexity and subtlety of marginal zone development. Importantly, these studies indicate that MZMs, MMMs, and sinus-lining cells can be independently regulated by TNF family cytokines during development.¹⁸ Furthermore, the involvement of discrete signaling pathways is becoming more apparent. For example, whereas selective retention of MZMs is noted in nuclear factor- κ B2-deficient mice,^{9,12} selective loss of MZMs is seen in Bcl-3-deficient mice.⁸ We suggest that this independent regulation is also likely to be mirrored in how these various cells behave in response to chronic inflammatory stimuli, such as is the case for TNF- α in this study. Thus, we anticipate that other chronic infections may lead to distinct patterns of marginal zone remodeling, driven by potentially distinct cytokine-mediated pathways. Our own ongoing studies in mice lacking various transcription factors hope to shed further light on the downstream events leading to MZM loss during *L. donovani* infection that are mediated through TNF- α .

The mechanism by which TNF- α mediates loss of MZMs remains to be determined. TNF- α may directly induce apoptosis in MZMs.⁴⁷ Alternatively, in the presence of TNF- α , MZMs may become more susceptible to lysis by expanding numbers of parasites within their phagolysosomal compartments. Both of these proposed mechanisms would require that MZMs are selectively more sensitive to these effects of TNF- α and/or parasite growth than similarly infected MMMs. Attempts to isolate these discrete macrophage populations and study their responses to TNF- α and to infection *in vitro* are hampered not only by difficulties in isolation per se but by the changes in macrophage function that inevitably accompany removal from their local matrix. Alternatively, TNF- α may cause emigration of MZMs from the spleen, a possibility supported by the observation that carbon preloaded into MZMs before infection, is not found in this organ at later times after infection (as might be expected if apoptotic MZMs were phagocytosed by other spleen resident macrophages). TNF- α is also an important mediator of tissue matrix metalloproteinase activity, and these molecules have been described to be involved in other tissue remodeling events controlled by excess TNF- α .^{48,49}

When MZMs are depleted from normal mice with liposome-encapsulated drugs, they begin to repopulate the

spleen marginal zone within 28 days,⁵⁰ yet our data indicates that during chronic *L. donovani* infection, MZMs are not recovered throughout a 166-day period (Figure 2). In contrast, MMMs have a replacement time of ~14 days,⁵⁰ and although they are reduced in number by day 56 after infection, they substantially recover by day 180 after infection (Figure 3). However, we have previously shown that during chronic *L. donovani* infection, myelopoietic activity in the spleen is significantly raised, with both a 20-fold increase in the number of colony forming units-granulocyte, monocyte (CFU-GM), and a dramatic increase in the proportion of these cells in S phase.⁵¹ The continued absence of MZMs throughout such an extended period suggests that the microenvironment is no longer appropriate for their development, that they are out-competed for space in the marginal zone by other myeloid populations, or that their net replacement is slower than their removal. Further investigations addressing these various possibilities are currently underway.

The selective loss of MZ macrophages may have important implications for understanding the pathogenesis of human VL. MZMs are specialized for the uptake of polysaccharides,⁷ and loss of this activity may allow increased systemic spread of encapsulated bacteria in humans infected with *L. donovani*. Indeed, secondary bacterial infections are responsible for significant mortality in such patients.²⁶ In addition, the disruption to lymphocyte migration is likely to contribute to the immunocompromised status of patients with VL. Our data also provide an alternative explanation of the observed interaction between chemotherapy and cellular immunity. Effective antimony therapy has long been recognized as T cell-dependent, and it has been assumed that this relates to co-operation between antimony drugs and the cytokines involved in macrophage effector function.⁵² However, previous clinical studies have shown that active disease is accompanied by the loss of CD45RO⁺ memory T cells and that this process is reversed by chemotherapy.⁵³ Furthermore, high rates of apoptotic T cell death have also been observed in experimental models.⁵⁴ Together, these findings suggest that replenishment of the antigen-specific effector/memory T cell pool is a prerequisite to cure from VL, and we suggest that chemotherapy contributes to disease resolution by enhancing the capacity of naive T cells to migrate to an appropriate environment for activation by dendritic cells.^{55,56}

The identification of TNF- α as a major mediator of marginal zone remodeling during *L. donovani* infection may allow for the design of novel therapies that preserve MZMs, as an adjunct to conventional chemotherapy. Significantly, high circulating levels of TNF- α are a hallmark of clinical VL,^{57–59} and recent genetic studies suggest that a TNF- α promoter polymorphism associated with high levels of TNF- α transcription is associated with progression to VL (Mary Wilson, personal communication). However, given the critical role of TNF- α in the resolution of hepatic infection in murine models of VL,⁴⁶ the balance between controlling infection and preventing pathology will have to be given careful consideration.

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