SIGNR1-Negative Red Pulp Macrophages Protect against Acute Streptococcal Sepsis after *Leishmania donovani*-Induced Loss of Marginal Zone Macrophages

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Marginal zone macrophages in the murine spleen play an important role in the capture of blood-borne pathogens and are viewed as an essential component of host defense against the development of pneumococcal sepsis. However, we and others have previously described the loss of marginal zone macrophages associated with the splenomegaly that follows a variety of viral and protozoal infections; this finding raises the question of whether these infected mice would become more susceptible to secondary pneumococcal infection. Contrary to expectations, we found that mice lacking marginal zone macrophages resulting from *Leishmania donovani* infection have increased resistance to *Streptococcus pneumoniae* type 3 and do not develop sepsis. Using biophotonic imaging, we observed that pneumococci are rapidly trapped in the spleens of *L. donovani*-infected mice. By selective depletion studies using clodronate liposomes, depleting monoclonal antibodies specific for Ly6C/G and Ly6G, and CD11c-DTR mice, we show that the enhanced early resistance in *L. donovani*-infected mice is entirely due to the activity of SIGNR1[−] red pulp macrophages. Our data demonstrate, therefore, that the normal requirement for SIGNR1⁺ marginal zone macrophages to protect against a primary pneumococcal infection can, under conditions of splenomegaly, be readily compensated for by activated red pulp macrophages. (Am J Pathol 2009, 175:1107–1115; DOI: 10.2353/ajpath.2009.090258)

the murine spleen, blood enters the marginal zone, a distinct anatomical microenvironment dominated by two macrophage populations: the marginal zone macrophages (MZMs) that face the red pulp and the marginal metallophilic macrophages (MMMs) that face inwards to the white pulp (reviewed in Ref. ¹). The marginal zone is therefore an important site for rapid capture of blood-borne bacterial,^{2,3} viral,⁴ and protozoal⁵ pathogens and although MZMs are most often attributed with the greatest phagocytic activity, MMMs also display this property.⁵

MZMs and MMMs express distinct surface phenotypes that serve to shape their function. MMMs are notable for their high constitutive expression of sialoadhesin (CD169, Siglec-1),^{6,7} whereas MZMs are characterized by their expression of the C-type lectin SIGNR1 (the murine homolog of human dendritic cell [DC]-specific intracellular adhesion molecule-grabbing nonintegrin [SIGN])⁸ and the scavenger receptor MARCO. SIGNR1 binds streptococcal capsular polysaccharides⁹ and intact *S. pneumoniae*^{3,10} *in vivo*, and expression of SIGNR1 on MZMs has been suggested to be essential for effective clearance of pneumococci.^{3,10} The mechanism by which SIGNR1 enhances protection, however, remains unclear. It is likely that binding of bacteria via SIGNR1 increases phagocytosis by MZMs.³ As an alternative, SIGNR1-mediated signals may be involved in increasing early anti-pneumococcal phosphorylcholine IgM responses from marginal zone B cells.¹¹ However, several studies suggest that SIGNR1 does not contribute to IgM or IgG responses against phosphorylcholine or capsular polysaccharides.^{3,12} Nevertheless, a consensus has emerged that SIGNR1-mediated bacterial binding in the marginal zone is central to a rapid protective response against blood borne infection with *S. pneumoniae*.

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The spleen has an important role in homeostasis and disease and acts as a filter through which all blood passes.¹ In

Splenomegaly arises from a variety of infectious and noninfectious conditions, including acute and chronic viral, bacterial, and parasitic disease,^{13–16} as well as neoplastic¹⁷ and autoimmune¹⁸ disease. When it has been examined, the integrity of the marginal zone is, to a greater or lesser extent, compromised by this process. For example, during the development of splenomegaly associated with chronic infection with *Leishmania donovani*, MZMs are progressively lost from the spleen, with only limited remodeling of the MMMs and marginal sinus.¹⁹ Given the important functional role of MZMs detailed above, we therefore hypothesized that loss of MZMs and the breakdown of the marginal zone may increase the susceptibility of *L. donovani*-infected animals to secondary blood-borne infections. To test this hypothesis, mice with chronic *L. donovani* infection were co-infected with *S. pneumoniae*. However, contrary to expectations, *L. donovani*-infected mice exhibited enhanced resistance, associated with an increased ability to clear bacteria from the bloodstream. We demonstrate that in the absence of an intact marginal zone or other SIGNR1-expressing cells, red pulp macrophages take on a compensatory role in host defense, and thus the dependence on SIGNR1 observed during primary pneumococcal infection is not evident under conditions of co-infection.

Materials and Methods

Mice and *L. donovani* Infection

BALB/c, C57BL/6J, and CD11c-DTR mice²⁰ on a B6 background (a generous gift of Dimitris Kioussis, National Institute for Medical Research) were bred in house. Mice were infected with 5×10^7 amastigotes of the LV9 strain of *L. donovani* i.v. at 6 to 8 weeks old and used at days 35 to 56 after infection, during the chronic phase of splenic infection. Animal experiments were performed with local ethical approval and under UK Home Office License.

S. pneumoniae Infection and in Vivo Imaging

Bioluminescent, luciferase-expressing *S. pneumoniae* strain Xen10 (serotype 3, A66.1 derivative, Xenogen Corp. Hopkinton, MA)²¹ was grown from frozen stocks to log phase in brain heart infusion broth. Bacteria were washed, and the concentration was determined spectrophotometrically and resuspended in PBS as appropriate. Mice were challenged i.v. with 1 to 5×10^6 colony forming units, and bacterial growth was followed in individual mice using the IVIS Imaging 100 system (Xenogen Corp.). The dose leads to a rapid systemic infection in naive mice, which is generally lethal at 20 to 48 hours after challenge. Luminescence (photons/second/cm²/sr) was quantified using LivingImage software (version 2.50, Xenogen Corp.), and statistical significance was determined with Student's *t*-test. For subsequent immunofluorescent localization, *S. pneumoniae* were labeled with the dye PKH26

(Sigma, Poole, UK) according to the manufacturer's instructions.

Serum Transfer and in Vivo Depletions

For serum transfer, whole blood was taken from age-matched control mice or mice at day 35 of *L. donovani* infection, and serum was prepared. Naive recipients were given 300 μ l of serum i.v. 4 hours before the *S. pneumoniae* challenge.

For depletion of tissue macrophages mice were treated 24 hours before *S. pneumoniae* challenge with 200 μ l i.v. of a suspension of clodronate liposomes. Clodronate was a gift of Roche Diagnostics GmbH (Mannheim, Germany). It was encapsulated in liposomes as described earlier.²² Antibody-mediated depletions were performed using anti-Ly6C/G (RB6-8C5, 500 μ g/mouse), anti-Ly6G (1A8, 200 μ g/mouse), or MAC4 (isotype control, 500 μ g/mouse). Mice were treated with antibody i.p. 24 hours before *S. pneumoniae* challenge. To deplete CD11c⁺ cells, including splenic DCs, CD11c-DTR mice were treated with diphtheria toxin (DTx) (4 ng/g b.wt., Sigma) 24 hours before *S. pneumoniae* challenge. As a control group, wild-type C57BL/6 mice were similarly treated with DTx. No effect on CD11c⁺ populations or MZMs/MMMs was observed in this group. Depletion of the expected populations in the spleen was confirmed by immunofluorescence microscopy and/or flow cytometry in each case (see Results).

Flow Cytometry and Immunofluorescence

Spleen samples were prepared for flow cytometric analysis as described previously.²³ All samples were treated with anti-CD16/32 (clone 2.4G2, eBioscience, San Diego, CA) before staining for flow cytometry. The following antibodies were used (from eBioscience unless stated otherwise): N418 (anti-CD11c); M1/70 (anti-CD11b); FA11 (anti-CD68, Acris Antibodies, Hiddenhausen, Germany); BM8 (anti-F4/80); 7/4 (anti-7/4 antigen, Caltag Laboratories, Burlingame, CA); M5/114 (anti-MHCII); RB6-8C5 (anti-Ly6C/G); ED3 (anti-CD169; AbD Serotec, Oxford, UK); ERTR9 (anti-SIGNR1, Bachem, St. Helens, UK); and appropriate isotype controls. Samples were acquired using a CyanADP flow cytometer (Beckman Coulter, High Wycombe, UK) and analyzed using Summit v4.3 software (Beckman Coulter).

For immunofluorescence microscopy, tissue samples were snap-frozen in Tissue-Tek OCT (VWR, Loughborough, UK), and 7- μ m sections were cut. Staining was performed as described previously,²⁴ using the antibodies listed above. In some cases nuclei were visualized by the inclusion of 4,6-diamidino-2-phenylindole in the final incubation. Samples were mounted in ProLong Gold (Invitrogen, Paisley, UK) and imaged using a Zeiss Axioplan LSM 510 confocal microscope as single optical slices (0.8 to 1.0 μ m). Images were analyzed using Zeiss LSM Image Browser software v4 and Adobe Photoshop CS.

Figure 1. Progression of acute *S. pneumoniae* Xen10 infection in control and *L. donovani*-infected mice. **A:** Control mice (left panels) or mice at day 42 after *L. donovani* infection (right panels) were challenged with bioluminescent *S. pneumoniae* Xen10 i.v. and monitored for total body luminescence. Colored scale bars denote luminescence (photons/second/cm²/sr). Note the differing scale for top and bottom panels. Red squares and ellipses in top panels indicate areas used to measure torso and splenic luminescence, respectively, in subsequent figures. Images are representative of all imaging experiments. **B:** Graph shows survival of previously naive mice ($n = 30$; solid line) and *L. donovani*-infected mice ($n = 20$; broken line) over the first 20 hours after an i.v. challenge with *S. pneumoniae* Xen10. Survival is significantly enhanced in *L. donovani*-infected mice ($P < 0.0001$; two-tailed log-rank test). **C:** Control and *L. donovani*-infected mice were infected i.v. with *S. pneumoniae* Xen10. Bioluminescence of the spleen and torso of individual mice were measured at various time points after infection. **Top:** bioluminescence of individual control (closed circles) and *L. donovani*-infected (open circles) mice. **Bottom:** mean $\pm$ SD at each time point. * $P < 0.05$ by Student's *t*-test. Graphs are representative of three individual experiments.

Results

Chronic *L. donovani* Infection Protects against Streptococcal Challenge

We examined whether the disrupted splenic architecture associated with chronic *L. donovani* infection altered host susceptibility to blood-borne pathogens using a model of acute streptococcal sepsis. Xen10 is a constitutively bioluminescent *S. pneumoniae* of a serotype (type 3) known to bind SIGNR1.⁹ Xen10 was injected i.v., and the progression of infection was followed *in vivo* (Figure 1A). Surprisingly, mice with chronic *L. donovani* infection were significantly more resistant to *S. pneumoniae* infection than age-matched control mice, with significantly enhanced 24-hour survival ($P < 0.0001$) (Figure 1B).

Bioluminescent imaging of *S. pneumoniae* demonstrated that in control mice, splenic uptake of pneumococci was rapidly followed by dissemination with obvious septicemia from 2 hours after infection onward (Figure

1A, left panels). In contrast, in *L. donovani*-infected mice, pneumococci were readily sequestered within the enlarged spleen, but failed to disseminate thereafter (Figure 1A, right panels). These qualitative observations were validated by quantitative measurements of bioluminescent signal from multiple mice per time point, evaluating either the signal derived from the spleen alone or the entire torso. From as early as 1 hour after challenge, *L. donovani*-infected mice showed evidence of enhanced resistance (Figure 1C). Together these data show that, contrary to expectation, *L. donovani* infection increased, rather than decreased, host resistance to co-infection with this acute bacterial infection.

Protection Does Not Involve Serum Factors

First, because *L. donovani* infection in mice is associated with hypergammaglobulinemia,²⁵ we determined whether serum from mice with chronic *L. donovani* infection could

Figure 2. Serum from *L. donovani*-infected mice does not enhance clearance of *S. pneumoniae*. **A** and **B**: Previously naive mice were given serum from naive control mice or from mice at day 42 of *L. donovani* infection. At 4 hours after i.v. serum transfer each group was challenged i.v. with *S. pneumoniae* Xen10, and bioluminescence was measured at various time points. Images are representative of bioluminescence observed at 4 hours after infection in mice given **(A)** naive serum and **(B)** serum from *L. donovani*-infected donors. **C** and **D**: Graphs show mean $\pm$ SD bioluminescence in the spleen and torso of mice given naive serum (closed circles) or *L. donovani* serum (open circles). $n = 5$ /group. No significant differences between groups were observed at any time point ($P > 0.05$; Student's *t*-test).

transfer resistance to control mice. Serum from *L. donovani*-infected mice or from age-matched naive mice was transferred i.v. into naive recipients 4 hours before challenge with *S. pneumoniae* (Figure 2, A and B). No quantitative difference in splenic pneumococcal load (Figure 2C) or in pneumococcal dissemination (Figure 2D) was observed between those mice given naive serum and those given serum from chronic *L. donovani* infection. This result suggests that serum components do not contribute to the protective effect of *L. donovani* infection against pneumococcal sepsis.

SIGNR1[−] Red Pulp Macrophages Are Required for Anti-Pneumococcal Resistance

In the absence of any serum-associated protective effects, we tested whether protection against *S. pneumoniae* was due to increased phagocyte activity in the spleen of *L. donovani*-infected mice. The splenomegaly associated with *L. donovani* infection resulted in a significant increase in cellularity of the spleen ($3.53 \pm 0.15 \times 10^8$ cells/spleen, $n = 6$) by day 35 after challenge compared with that in age-matched control mice ($1.01 \pm 0.09 \times 10^8$ cells/spleen, $n = 6$; $P < 0.0001$). By flow cytometry, red pulp macrophages in both naive and *L. donovani*-infected mice can be defined as CD68^{HI}F4/80⁺CD11c[−]CD11b[−]7/4[−] autofluorescent cells with distinct forward and side scatter properties. These cells comprised a mean of $2.7 \pm 0.6\%$ of total splenocytes in naive mice or $2.7 \pm 0.5 \times 10^6$ red pulp macrophages/spleen. At day 35 of *L. donovani* infection red pulp macrophages were significantly increased in both proportion

($5.1 \pm 0.2\%$, $P = 0.0001$) and absolute number ($17.6 \pm 2.0 \times 10^6$ red pulp macrophages/spleen, $P < 0.0001$). Thus, *L. donovani* infection induces a substantial increase in the absolute number of red pulp macrophages. In the context of anti-pneumococcal resistance, the role of red pulp macrophages has not been examined previously.

To determine the contribution of splenic macrophages to anti-pneumococcal resistance, we first treated mice i.v. with 200 μ l of clodronate liposomes, 24 hours before *S. pneumoniae* challenge. This regimen primarily depletes tissue macrophages but can also affect other populations including circulating monocytes.²⁶ In our hands, liposome treatment of naive mice depleted most red pulp macrophages in the spleen, with a few CD68⁺ cells remaining. In addition, this regimen also depleted most MZMs and some MMMs, as assessed by immunofluorescence (Figure 3B). Treatment of naive mice with clodronate liposomes significantly increased susceptibility to *S. pneumoniae* and was associated with increased bacterial burden at early time points ($P = 0.0002$ at 2 hours compared with control) (Figure 3A). In *L. donovani*-infected mice, clodronate liposome treatment resulted in the depletion of most, but not all, red pulp macrophages in the spleen and of some MMMs (Figure 3B). However, this partial depletion, primarily of red pulp macrophages, was sufficient to render *L. donovani*-infected mice acutely susceptible to *S. pneumoniae* (Figure 3A). Liposome-treated *L. donovani*-infected mice showed significantly increased bacterial load, as demonstrated by increased bioluminescence, with associated sepsis at 4 hours after *S. pneumoniae* challenge ($P = 0.03$). Moreover, no liposome-treated *L. donovani*-infected mice survived to the 18-hour time point, in contrast with the 100% survival of untreated *L. donovani*-infected mice at this time.

Because SIGNR1 expression has been suggested to be critical for controlling early pneumococcal infection,^{3,10,11} we examined whether *L. donovani* infection induced SIGNR1 expression on red pulp macrophages or on splenocytes other than MZMs. Examination of frozen splenic tissue sections by immunofluorescence indicated that SIGNR1 expression was indeed restricted to the few remaining MZMs seen in the spleens of *L. donovani*-infected mice (Figure 3B). To confirm this observation, we compared SIGNR1 expression on splenocytes from naive mice and from *L. donovani*-infected mice by flow cytometry. With this approach, negligible low levels of SIGNR1 were detected on red pulp macrophages (CD68^{HI}CD169[−], large granular cells) and splenic DCs (CD68[−]CD11c^{HI}MHCII^{HI} cells) in naive mice, whereas a subset of SIGNR1^{HI} cells was present among CD68[−]CD169[−] large, granular cells in naive spleens (Figure 3, C–E, left panels). These latter cells (Figure 3E, R1-gated) probably correspond to MZMs, having high SIGNR1 expression, although this possibility was not definitively confirmed. *Leishmania* infection did not induce SIGNR1 expression by CD68^{HI}CD169[−] red pulp macrophages, and neither were alternate SIGNR1-expressing populations present in the spleen (Figure 3, C–E, right panels, $n = 5$ and data not shown). Therefore, although a contribution of MMMs cannot be formally excluded, these experiments sug-

Figure 3. Depletion of red pulp macrophages reduces *S. pneumoniae* clearance in *L. donovani*-infected mice. **A:** Naive mice or mice at day 42 after *L. donovani* infection were treated i.v. with clodronate liposomes 24 hours before *S. pneumoniae* challenge. The graph shows mean $\pm$ SD torso bioluminescence after an *S. pneumoniae* challenge in untreated naive mice (closed circles) or naive mice treated with clodronate liposomes (closed squares) and in *L. donovani*-infected mice (open circles) or *L. donovani*-infected mice treated with clodronate liposomes (open squares). $n = 5$ /group. Numbers indicate P values against the relevant control group at the selected time point. $^+$ One survivor only at 4 hours. * All mice succumbed to infection before the 18-hour time point. **B:** Cryosections of spleen from naive control and clodronate liposome-treated mice (**top panels**) or from *L. donovani*-infected control and clodronate liposome-treated mice stained for expression of CD68 (green), CD169 (red), and SIGNR1 (blue). Original magnification, $\times 200$. **C:** Splenic populations from naive mice (**left panels**) or *L. donovani*-infected mice (**right panels**) stained with antibody against SIGNR1 or with isotype control antibody. Red pulp macrophages (RPM) were defined as CD68⁺CD169⁺FSC⁺SSC⁺ cells. DCs were CD11c⁺F4/80⁺ cells. **D:** Putative MZMs in naive spleens were SIGNR1⁺ cells (**E**) (R1-gated) within the CD68⁺CD169⁺FSC⁺SSC⁺ population.

gest that enhanced protection against pneumococcal sepsis observed in *L. donovani*-infected mice is mediated by red pulp macrophages and is independent of SIGNR1 expression.

Neutrophils and DCs Do Not Contribute to Early Host Resistance

Although i.v. clodronate liposome treatment primarily affects tissue macrophages,²⁷ it may also affect other phagocytic populations that may be involved in early anti-pneumococcal responses. To determine whether other phagocytes played a role in the enhanced resistance observed in *L. donovani*-infected mice, mice were first administered anti-Ly6C/G, which depletes both neutrophils and monocytes, or anti-Ly6G, which specifically depletes neutrophils.²⁸ The effects of anti-Ly6C/G treatment on splenic populations in naive mice is well characterized, including by our group.²⁹ To confirm the efficacy of treatment with anti-Ly6C/G and anti-Ly6G in the context of chronic *L. donovani* infection we examined spleens by immunofluorescence for expression of the 7/4 antigen, which is expressed by both neutrophils and monocytes. This examination confirmed the presence of large numbers of 7/4⁺ cells in *L. donovani*-infected spleens (Figure 4A) and the fact that both antibodies efficiently depleted 7/4⁺ cells *in vivo*. Based on these depletion studies, neutrophils comprised the majority of 7/4⁺ cells in the spleen, because very few 7/4⁺ cells remained after neutrophil-specific depletion (Figure 4A, right panel). Depletion of monocytes and neutrophils from control mice had no significant effect on bacterial load over the first 8 hours of *S. pneumoniae* infection. Previously naive mice left untreated, given control antibody, or depleted of monocytes and neutrophils progressed rapidly to sepsis, and no mice survived to 20 hours ($n = 5$ /group) (Figure 4C). Likewise depletion of monocytes and/or neutrophils from *L. donovani*-infected mice did not significantly increase the bacterial load in these groups over the first 8 hours after *S. pneumoniae* challenge (Figure 4D). At 20 hours, bacterial loads were increased in depleted mice compared with those in controls and significantly so for *L. donovani*-infected mice depleted of both neutrophils and monocytes ($P = 0.02$). However, in contrast with control mice, no *L. donovani*-infected mice depleted of monocytes and/or neutrophils had succumbed to infection by 20 hours after infection (Figure 4D).

We next examined resistance to *S. pneumoniae* in *L. donovani*-infected CD11cDTR mice with or without administration of DTx. Although DTx administration had no effect on cells in wild-type mice, it resulted in efficient depletion of splenic DCs in the CD11cDTR mice (Figure 4B). Moreover, DTx treatment also results in depletion of most CD169⁺ MMMs from the spleen, most likely because of erroneous expression of the transgene.³⁰ DTx treatment of *L. donovani*-infected mice, therefore, allows the examination of the effect of the combined depletion of splenic DCs and MMMs. Depletion of DCs and MMMs from *L. donovani*-infected mice before *S. pneumoniae*

Figure 4. Depletion of specific phagocyte populations does not impair early *S. pneumoniae* clearance in *L. donovani*-infected mice. Naive mice or mice at day 42 after *L. donovani* infection were treated i.v. with depleting antibodies against defined phagocytic cell populations 24 hours before *S. pneumoniae* challenge. **A:** Cryosections of spleen from *L. donovani*-infected control and anti-Ly6C/G- and anti-Ly6G-treated mice stained for expression of 7/4 antigen (green) and CD68 (red). Original magnification, $\times 200$. **B:** Cryosections of spleen from naive B6 mice treated 48 hours previously with DTx (**left**) and of spleen from *L. donovani*-infected B6.CD11cDTR control (**center**), and DTx-treated mice (**right**) stained for expression of CD169 (green) and CD11c (red). Original magnification, $\times 200$. **C–E:** Graphs show mean $\pm$ SD torso bioluminescence for control and antibody/DTx-treated groups after *S. pneumoniae* challenge. **C:** Naive untreated control mice (open squares) or naive mice pretreated with isotype control (closed squares) or anti-Ly6C/G (anti-neutrophil/monocyte; closed circles). **D:** *L. donovani*-infected mice pretreated with isotype control antibody (closed squares), with anti-Ly6C/G (closed circles), or with anti-Ly6C (anti-neutrophil; open circles). **E:** *L. donovani*-infected CD11cDTR mice (closed squares), and *L. donovani*-infected CD11cDTR mice pretreated with DTx (open squares). mAb, monoclonal antibody.

challenge had no effect on bacterial load or survival compared with controls (Figure 4E).

Together these targeted depletion experiments indicate that although neutrophils may contribute to protective responses at later time points, SIGNR1⁺ macrophages of the splenic red pulp have the most profound influence over early bacterial containment, clearance, and host survival in *L. donovani*-infected mice lacking MZMs.

Bacteria Rapidly Access the Splenic Red Pulp in *L. donovani*-Infected Mice

Although previous studies have focused on the uptake of *S. pneumoniae* by SIGNR1⁺ cells, it has been noted that some bacteria are found in the red pulp at early time points after challenge.^{3,11} To examine whether this was also the case in our model of infection, naive mice or *L. donovani*-infected mice were challenged with fluorescein isothiocyanate-dextran. At 30 minutes after challenge, we determined the localization of both SIGNR1⁺ cells (those capable of binding dextran^{3,31}) and bacteria in the spleen. As expected, in naive mice a significant proportion of bacteria were localized within dextran⁺ cells, indicating that at the dose used, dextran did not compete with bacteria for SIGNR1 binding (Figure 5A). We also confirmed that significant numbers of bacteria were present in fluorescein isothiocyanate-dextran[−] cells within the red pulp of naive mice. In *L. donovani*-infected

mice very few fluorescein isothiocyanate-dextran⁺ cells were observed. However, bacteria were found throughout the red pulp of these mice at 30 minutes after challenge (Figure 5B), demonstrating that circulating bacteria have rapid and widespread access to this compartment in *L. donovani*-infected mice, as well as to a lesser extent in naive mice.

Figure 5. Splenic distribution of *S. pneumoniae*. **A** and **B:** The localization of fluorescein isothiocyanate-dextran (**left**) and PKH26-labeled *S. pneumoniae* (**center**) are shown in spleens of naive (**A**) and *L. donovani*-infected (**B**) mice 30 minutes after i.v. administration. Composite images (**right**) are also shown. Original magnification, $\times 400$. **Insets:** arrows indicate colocalized dextran and *S. pneumoniae*, and circles indicate *S. pneumoniae* within dextran-negative populations.

Discussion

The specialized phagocyte populations of the splenic marginal zone are, through the expression of distinct pattern recognition receptors, considered indispensable mediators of protection against blood-borne pathogens. Notably, SIGNR1⁺ MZMs have been reported as essential for protection against systemic *S. pneumoniae* infection. Here, we show that the chronically inflamed, MZM-depleted splenic microenvironment induced by *L. donovani* infection confers enhanced resistance against *S. pneumoniae* challenge compared with that of the naive spleen. In the absence of SIGNR1⁺ MZMs, this protection is mediated by SIGNR1[−] red pulp macrophages.

Once *S. pneumoniae* access the bloodstream, host protection is thought to be dependent on the efficient SIGNR1-mediated binding of bacteria by MZMs as blood is filtered by the splenic marginal zone.^{1,3,9,10} On this basis, we had hypothesized that disruption of the marginal zone or reduction in numbers of MZMs might severely affect the ability to handle blood-borne *S. pneumoniae* infection. Surprisingly, however, *L. donovani*-infected mice exhibited enhanced protection against subsequent *S. pneumoniae* challenge. In this study, we used a bioluminescent strain of *S. pneumoniae* (Xen10) in which bioluminescence is dependent on and reflects continued active bacterial division²¹ to be able to directly and noninvasively examine bacterial localization during the course of infection. We found that during acute infection, naive mice with intact MZMs were unable to prevent the rapid onset of lethal sepsis (Figure 1). In contrast, *L. donovani*-infected mice seemed to readily capture bacteria in the spleen, as clearly demonstrated here by noninvasive imaging, resulting in local elimination of bacteria, a delayed progression to sepsis, and prolonged survival.

To our knowledge, this is the first study examining the effect of a range of cell depletions on the response of previously naive mice to pneumococcal sepsis. Not surprisingly, pretreatment of naive mice with clodronate liposomes significantly enhanced the onset of lethal sepsis, probably because of the almost complete loss of SIGNR1⁺ MZMs and of red pulp macrophages. However, in this acute sepsis model, depletion of circulating neutrophils (and other Ly6C⁺ populations, including plasmacytoid DCs and some monocytes) had no effect on the progression of disease. Any distinct contribution of plasmacytoid DCs was also excluded in a separate, plasmacytoid DC-specific depletion (data not shown). Together these results suggest that early splenic protection against pneumococcal sepsis in naive animals is mediated solely by resident splenic macrophage populations.

The strikingly increased capacity of *L. donovani*-infected mice to cope with an acute septic insult may be due to the systemic immunological environment engendered by chronic visceral leishmaniasis. For example, increased expression of interleukin-10 during chronic *L. donovani* infection³² may aid in limiting any toxic shock response.³³ On the other hand, although increased local interferon- γ and tumor necrosis factor- α may enhance macrophage bactericidal activity, the threshold of macrophage activation remains below that necessary to

achieve anti-leishmanial effects. In addition, human chronic *L. donovani* infection results in a strong humoral immune response to parasite antigens³⁴ and elevated circulating levels of acute-phase proteins.³⁵ The presence of potentially cross-reactive antibodies and higher levels of circulating acute-phase proteins, for example, may be beneficial in the early response to *S. pneumoniae*. However, the transfer of serum from *L. donovani*-infected mice into previously naive hosts did not reveal a role for circulating factors in the enhanced protection against *S. pneumoniae* (Figure 2). Serum transfer did not increase splenic trapping of *S. pneumoniae* at early time points, suggesting that opsonins within serum from *L. donovani*-infected mice do not make a significant contribution to the anti-pneumococcal response. This supports a cellular basis as the major protective mechanism in *L. donovani*-infected mice.

We have shown that *L. donovani*-infected mice have approximately sixfold more splenic red pulp macrophages than do naive control mice. Within the high-dose, rapidly lethal sepsis model used in this study the protective contribution of red pulp macrophages is clearly visible. Furthermore, in *L. donovani*-infected mice, the significant increase in the number of red pulp macrophages compensates for the absence of SIGNR1⁺ MZMs. Clodronate treatment of *L. donovani*-infected mice depletes red pulp macrophages and some MMMs (Figure 3B). Previous studies have not revealed a role for MMMs in the phagocytosis or clearance of *S. pneumoniae*.^{3,9,10} Therefore, although a role for MMMs in protection cannot be formally excluded, our results using clodronate treatment and DTx treatment of CD11c-DTR mice suggest that red pulp macrophages are the population responsible for the protection against *S. pneumoniae* infection observed in *L. donovani*-infected mice (Figure 3A). Although clodronate depletion in *L. donovani*-infected mice is relatively inefficient (Figure 3B), most likely as a result of the splenomegaly, it was nevertheless sufficient to render these individuals acutely susceptible to *S. pneumoniae* infection.

We confirmed the specific importance of red pulp macrophages in restricting early bacterial growth and dissemination by selectively depleting other phagocyte populations. Neither neutrophils nor monocytes were required to limit bacterial numbers over the first 8 hours after *S. pneumoniae* challenge in *L. donovani*-infected mice. Only at 8 to 20 hours after challenge do neutrophils and monocytes, to a lesser extent, contribute significantly to antibacterial responses in these hosts (Figure 4D). Human visceral leishmaniasis causes cellular changes not seen in the mouse model, including neutropenia, which contributes to secondary infections associated with the disease.³⁶ Our results suggest that neutropenia may have a far greater impact on longer-term susceptibility to secondary (blood-borne) infection in human visceral leishmaniasis than dysfunction of splenic macrophages.

SIGNR1-mediated binding by MZMs has been proposed as the critical mechanism conferring a protective early response against circulating pathogens and most strikingly against *S. pneumoniae*.^{3,11} Mice lacking SIGNR1 expression fail to clear *S. pneumoniae* from the blood-

stream,³ suggesting that other surface molecules are unable to compensate for SIGNR1 in the binding of *S. pneumoniae*. Moreover, these studies suggest that SIGNR1⁺ MZMs are themselves essential for pathogen clearance and cannot be functionally substituted by alternative phagocyte populations. However, *L. donovani*-infected mice have very few MZMs during the chronic stage of infection yet exhibit a strikingly enhanced level of protection against i.v. *S. pneumoniae* challenge, mediated by red pulp macrophages (Figure 3). With such importance placed on the role of SIGNR1, it is somewhat surprising that quantitative increases in red pulp macrophages that lack SIGNR1 expression (Figure 3, C and D) are sufficient to enhance protection. However, our data confirm that *S. pneumoniae* rapidly access the red pulp from the circulation even in mice with an intact marginal zone. In addition, we demonstrate that the splenic uptake of *S. pneumoniae* is by no means restricted to dextran-binding (SIGNR1⁺)³¹ cells such as MZMs in either naive or *L. donovani*-infected mice (Figure 5, A and B). Binding of *S. pneumoniae* to other populations, including red pulp macrophages, may be mediated via a range of pathogen recognition receptors, including Toll-like receptor 4, which binds pneumococcal pneumolysin,³⁷ and Toll-like receptor 2,³⁸ which recognizes lipoteichoic acid.³⁹

A recent study showed that the role of MZMs extends beyond phagocytic/antibacterial activity to encompass the initiation of *S. pneumoniae*-specific IgM responses.¹¹ Our data suggest a model in which red pulp macrophages make a significant contribution to early antibacterial activity, limiting bacterial dissemination although IgM responses are amplified by SIGNR1⁺ MZMs. In this model the absence of IgM responses in SIGNR1-deficient mice is ultimately responsible for susceptibility to infection. In the current system, *L. donovani*-infected mice treated with clodronate liposomes may, in some ways, equate to SIGNR1-deficient mice with regard to susceptibility. That is, they lack MZMs capable of binding *S. pneumoniae* and initiating IgM responses, while also lacking sufficient red pulp macrophages to delay the onset of disseminated infection.

Studies are ongoing to determine whether, in addition to an increase in red pulp macrophage number, *L. donovani* infection induces other effects beneficial to rapid anti-pneumococcal macrophage responses. Although systemic effects, eg, on serum factors, do not seem to be involved (Figure 2), the potential contribution of locally derived factors, such as opsonins, in the chronically infected *L. donovani* spleen remain to be investigated. On the other hand, *L. donovani* infection may result in pre-activation of red pulp macrophages, facilitating enhanced bactericidal activity. Intravenous pneumococcal challenge of *L. donovani*-infected mice induces a rapid oxidative burst response in the red pulp, which is not observed in similarly challenged naive animals (our unpublished data). The mechanisms behind this activity and the potential contribution of this and other macrophage-mediated antibacterial responses to the observed protective effect continue to be examined.

In summary, we have shown that SIGNR1⁺ cells rapidly take up *S. pneumoniae* in naive and *L. donovani*-infected mice and that bacterial uptake by SIGNR1⁺ red

pulp macrophages contributes significantly to early protection against bacterial dissemination.

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