Short Communication

Compartment-Specific Remodeling of Splenic Micro-Architecture during Experimental Visceral Leishmaniasis

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**Progressive splenomegaly is a hallmark of visceral
leishmaniasis in humans, canids, and rodents. In ex-
perimental murine visceral leishmaniasis, spleno-
megaly is accompanied by pronounced changes in
microarchitecture, including expansion of the red
pulp vascular system, neovascularization of the white
pulp, and remodeling of the stromal cell populations
that define the B-cell and T-cell compartments. Here,
we show that Ly6C/G$^+$ (Gr-1$^+$) cells, including neu-
trophils and inflammatory monocytes, accumulate in
the splenic red pulp during infection. Cell depletion
using monoclonal antibody against either Ly6C/G$^+$
(Gr-1; RB6) or Ly6G$^+$ (1A8) cells increased parasite
burden. In contrast, depletion of Ly6C/G$^+$ cells, but
not Ly6G$^+$ cells, halted the progressive remodeling of
Meca-32$^+$ and CD31$^+$ red pulp vasculature. Strikingly,
neither treatment affected white pulp neovasculariza-
tion or the remodeling of the fibroblastic reticular cell
and follicular dendritic cell networks. These findings
demonstrate a previously unrecognized compart-
ment-dependent selectivity to the process of splenic
vascular remodeling during experimental murine vis-
ceral leishmaniasis, attributable to Ly6C$^+$ inflamma-
tory monocytes.** *(Am J Pathol 2011, 179:23–29; DOI:
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Leishmaniasis, caused by protozoan parasites (genus
Leishmania), is a major neglected disease in resource-
poor countries. The species *L. donovani* and *L. infantum*
are responsible for visceral leishmaniasis, the most se-

vere form of the disease, which if untreated is almost
universally fatal.[1] In mouse models, as with human and
canine disease, the spleen is the site of chronic infec-
tion.[2] As infection progresses, marked splenomegaly de-
velops, associated with extensive remodeling of the lym-
phoid tissue microarchitecture. Elements of the marginal
zone are either lost or disrupted in their spatial organiza-
tion,[3] and follicular dendritic cells (FDCs) and fibroblastic
reticular cells (FRCs), the key stromal elements that un-
derpin B cell follicle integrity and form the T-cell zone
conduit system, respectively,[4,5] are progressively de-
nuded.

More recently, we and others have demonstrated that,
in murine experimental visceral leishmaniasis (EVL)[6] and
in canine VL,[7] there are pronounced changes to the
splenic vasculature that accompany splenomegaly. No-
tably, in EVL, Meca-32$^+$ red pulp vessels are greatly
enlarged and show signs of angiogenesis. In the white
pulp, neovascularization leads to the appearance of nu-
merous CD31$^+\alpha$SMA-1$^+$ vessels that develop coincident
with a breakdown in the organization of the FRC and FDC
networks.[6] Studies in wild-type mice treated with neutral-
izing antibodies and in tumor necrosis factor (TNF)-defi-
cient mice have implicated the proinflammatory cytokine
tumor necrosis factor (which is overexpressed by mac-
rophages and many other cells during EVL) as a mediator
of white pulp stromal cell remodeling.[3,4] However, little is

known about the regulation of vascular remodeling within the red pulp or neovascularization in the white pulp.

Both neutrophils and inflammatory monocytes have recently attracted much interest in the study of leishmaniasis, by acting as a host cells during infection[8-10] and by playing a variety of regulatory roles that affect late stages of both visceral and cutaneous disease.[10–12] More recently, neutrophils that express matrix metalloproteinase 9 (MMP-9),[13–15] as well as inflammatory monocytes,[16] have been implicated in the regulation of angiogenesis in a variety of experimental models of disease. In the context of EVL, however, it is currently unknown whether either cell population plays a role in the splenic tissue remodeling that occurs during progressive disease.

In the present study, therefore, we sought to determine the effects of depletion of these cells on the progressive changes to splenic architecture that are characteristic of this disease. With use of flow cytometry and confocal microscopy, we show that Ly6C/G^{high} neutrophils and Ly6C/G^{int} inflammatory monocytes are located within the splenic red pulp and that their number increases during infection. Depletion of either Ly6C/G$^{int/high}$ cells [using Gr-1 monoclonal antibody (mAb) RB6] or Ly6G$^+$ cells (using mAb 1A8) from day 21 to day 28 of infection increased splenic parasite load, but these treatments had distinctly different effects on splenic remodeling. Gr-1 administration led to a selective arrest in red pulp vascular remodeling, whereas neovascularization and the disruption of stromal cell networks in the white pulp continued unabated. In contrast, administration of mAb 1A8 had no effect on any aspect of splenic remodeling that we measured. These data demonstrate for the first time that the regulation of splenic vascular remodeling during EVL occurs in a strictly compartmentalized manner and is independent of mechanisms that control parasite burden.

Materials and Methods

Mice and Infection

Female C57BL/6 mice, 6 to 8 weeks old, were obtained from Charles River UK (Margate, UK) and were housed under specific-pathogen-free conditions. *L. donovani* (LV9) amastigotes were obtained from the spleens of infected Syrian hamsters, as described previously.[17] Mice were injected with 3×10^7 amastigotes i.v. via the lateral tail vein. At day 21 and day 24 after infection, mice were administered 0.2 mg i.p. of Ly6C/G-specific Gr-1 (RB6-8C5) or 0.25 mg Ly6G-specific (1A8) mAbs or their isotype control antibodies (AFRC Mac-5 or Mac-4, respectively). Mice were sacrificed by cervical dislocation at day 28 and spleens were removed for analysis. All experiments were approved by the institutional ethics committee and were performed under U.K. Home Office license.

IHC

Spleen sections (10-μm thick) cut from spleens of both uninfected and infected mice were fixed in ice-cold ac-

etone or 2% paraformaldehyde at room temperature for 15 minutes. Paraformaldehyde-fixed tissue sections were then blocked and permeabilized by 0.1% bovine serum albumin (w/w), 0.1% (v/v) saponin in PBS for 1 hour at room temperature. For MMP-9 localization, sections were double-stained with biotinylated rat anti-MMP-9 (R&D Systems, Minneapolis, MN) and with allophycocyanin-conjugated Gr-1, F4/80, or CD169 (eBioscience, San Diego, CA) primary antibodies, followed by streptavidin Alexa Fluor 488 or streptavidin Alexa Fluor 647. Slides were washed three times with 0.1% (v/v) saponin in PBS. The sections were dehydrated after staining, mounted with antifade solution (Molecular Probes, Eugene, OR), and visualized using a Zeiss Axioplan LSM 510 confocal microscope. For the assessment of vascular changes, sections were stained using biotinylated CD31 (eBioscience) or purified Meca-32 (BD Pharmingen, San Diego, CA) followed by Alexa Fluor 488 streptavidin and fluorescein isothiocyanate goat anti-rat antibody (both from Invitrogen, Carlsbad, CA). Localization of Gr-1$^+$ and F4/80$^+$ cells was performed using biotinylated mAb followed by streptavidin conjugated to Alexa Fluor 546 and Alexa Fluor 647 conjugated mAb, respectively. Data were analyzed by quantitative morphometry, as described previously.[6]

In Situ *Zymography*

In situ zymography with fluorescein isothiocyanate-labeled dye-quenched gelatin (Enz Chek; Molecular Probes, Eugene, OR) was used to determine the localization of MMP-9 activity in spleen sections. Unfixed sections (8-μm thick) were thawed, preincubated in PBS at room temperature, and incubated with PBS containing 40 μg/mL dye-quenched gelatin (1 mg/mL) in a humidified chamber overnight. At the end of the incubation period, sections were washed three times with PBS and then were dehydrated and mounted with antifade solution. As controls, a general MMP inhibitor, 1,10-phenanthroline (10 mmol/L; Sigma-Aldrich, St. Louis, MO), was added to the PBS/dye-quenched gelatin mixture and applied to frozen cryostat sections. Nonspecific serine protease activity was blocked using phenylmethylsulfonyl fluoride (Sigma-Aldrich). Images were acquired with a Zeiss Axioplan LSM 510 confocal microscope.

Flow Cytometry and Intracellular Enzyme Detection

Splenic cells from naïve or infected mice were homogenized in RPMI and cells were counted on hemocytometer. Then 1 to 2×10^6 cells per tube were resuspended in ice-cold PBS buffer consisting of 5 mmol/L EDTA, 1% fetal calf serum, and 1% NaNO$_3$. All stainings were performed on ice. Splenic cells were surface stained with phycoerythrin or allophycocyanin conjugated Gr-1, Ly6G-phycoerythrin, Ly6C-fluorescein isothiocyanate, CD11b-Pacific blue, CD11c phycoerythrin-Cy7, and MHCII-allophycocyanin. GR-1 stained
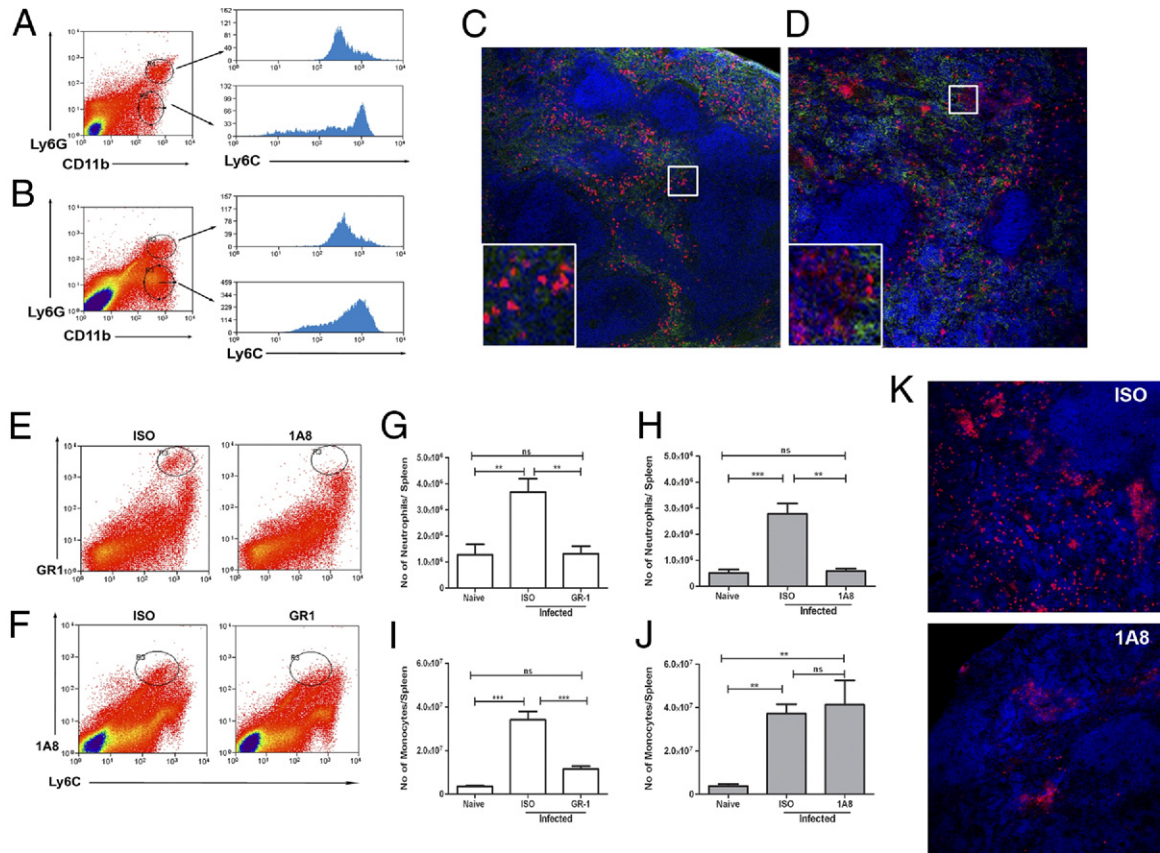
Figure 1. Identification of Ly6C/G$^+$ cells in EVL. Spleens from *L. donovani* infected mice were removed at day 28 after infection and analyzed alongside spleens from naïve mice by flow cytometry and by IHC. **A** and **B:** Spleen cells from naïve (**A**) and infected (**B**) mice were stained with Ly6G and CD11b and the expression of Ly6C was determined on neutrophils (**upper** histograms) and monocytes/DC (**lower** histograms). **C** and **D:** Sections from naïve (**C**) and infected (**D**) mice were stained for Ly6C/G (Gr-1; red) and F4/80 (green) and counterstained with DAPI (blue). Original magnification, ×100. **Insets** show boxed areas at higher magnification: Gr-1^{bright} neutrophils (**C**) and a nest of F4/80$^-$Gr-1^{dull} cells with two neutrophils in close proximity (**D**). Original magnification, ×630. **E:** Spleen cells from infected mice treated with 1A8 or isotype control antibody were stained with Gr-1 and Ly6C. Gr-1$^+$Ly6C$^+$ neutrophils are highlighted in **oval** gated regions. **F:** Spleen cells from infected mice treated with Gr-1 or isotype control antibody were stained with 1A8 and Ly6C. 1A8$^+$Ly6C$^+$ neutrophils are highlighted in **oval** gated regions. **G–J:** Absolute numbers of splenic neutrophils (**G** and **H**) and monocytes/DC (**I** and **J**) in spleens of naïve mice and infected mice were determined using Ly6G (1A8) and Ly6C for Gr-1-depleted mice (**G** and **I**) and using Ly6C and Gr-1 for 1A8-depleted mice (**H** and **J**). Data represent means ± SEM from 7 mice per group, pooled from two independent experiments for Gr-1 depletion and 4 mice per group from one experiment for 1A8 depletion. Similar results were seen in an additional experiment in which 1A8-depleted mice were analyzed using CD11b and Ly6C (data not shown). **P < 0.01, ***P < 0.001. ns, not significant. Peripheral blood neutrophils were depleted by 91 ± 4% after 1A8 and 98 ± 0.5% after Gr-1 depletion (data not shown). **K:** Spleen sections from infected mice administered either isotype control (ISO) or 1A8 were stained for Gr-1 (red) and DAPI (blue). Original magnification, ×200.

cells were washed twice with EDTA/fetal calf serum/ NaNO$_3$, resuspended in the same buffer, and incubated 15 minutes on ice with 2% paraformaldehyde. Fixed cells were then resuspended in PBS/EDTA/fetal calf serum/NaNO$_3$ containing 0.5% saponin and intracellularly labeled with biotinylated anti-MMP-9. Biotinylated antibodies were visualized with fluorescein isothiocyanate-streptavidin. Background staining was judged from appropriate isotype control mAbs. Flow cytometric analysis was performed using a CyAn flow cytometry system (Beckman Coulter, High Wycombe, UK) and Summit 4.0 software.

Statistical Analysis

Statistical analysis was performed using a nonpaired Student's *t*-test and two-way analysis of variance. *P* < 0.05 was considered significant. All experiments were repeated independently at least twice.

Results

CD11b$^+$Ly6C$^+$ Cells Accumulate in the Spleen During L. donovani *Infection*

In the spleen of naïve mice, neutrophils can be distinguished from monocytes/DC using a combination of CD11b and Ly6G (Figure 1A). Within the CD11b$^+$Ly6G$^-$ monocyte/DC population, considerable heterogeneity was found in expression of Ly6C, as has been observed by others.[18] During infection with *L. donovani*, the relative balance of neutrophils to monocyte/DC shifts from a ratio of approximately 1:1 to approximately 1:6, reflecting recruitment of inflammatory CD11b$^+$Ly6G$^-$ monocytes/DC cells that are more homogeneous for Ly6C expression (Figure 1B). By immunohistochemistry (IHC), two populations of Ly6C/G$^+$ cells were also readily distinguished (Figure 1, C and D), both localized within the red pulp. Ly6C/G^{bright} cells, almost invariably with polymorphonuclear appearance, were found
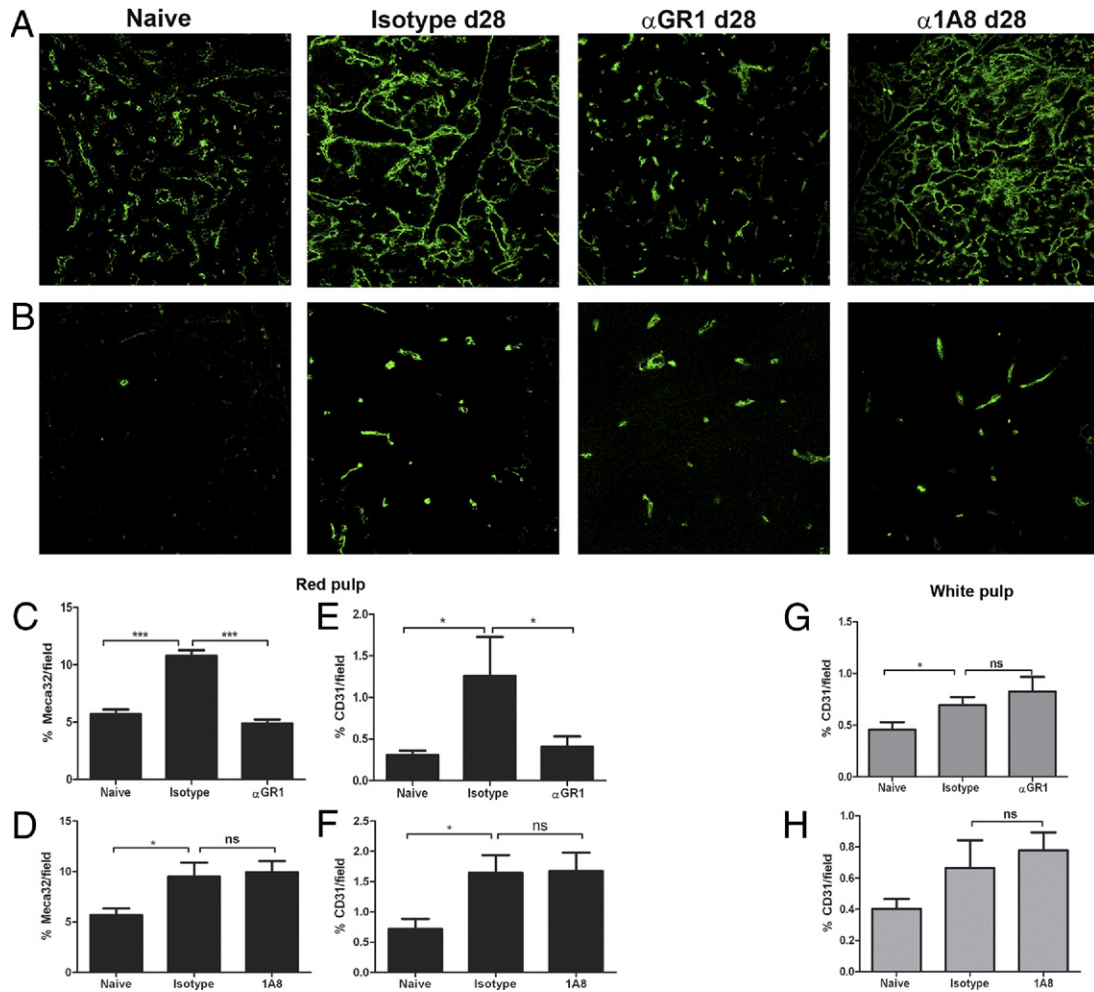
Figure 2. Ly6C^{+} inflammatory monocytes/DC promote vascular remodeling in the red pulp. Spleens from mice infected with *L. donovani* were removed at day 28 after infection and analyzed alongside spleens from naïve mice by IHC. **A** and **B:** Spleen sections from naïve mice and infected mice treated with isotype control, Gr-1, or 1A8 from day 21 to day 28 after infection were stained with Meca-32 (**A**) or with CD31 (**B**). Original magnification, ×200. **C–H:** Computer-assisted quantitative morphometry was performed to determine the expression of Meca-32 (**C** and **D**) and of CD31 (**E** and **F**) in the red pulp and also the expression of CD31 in the white pulp (**G** and **H**) of mice treated with Gr-1 (**C, E,** and **G**) or 1A8 (**D, F,** and **H**). Data were generated from multiple sections examined from at least 5 mice per group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

throughout the red pulp in naïve mice (Figure 1C), whereas most Ly6C/G[dull] cells were concentrated in the subcapsular red pulp and therefore likely represent the resident splenic monocyte pool.[18] In contrast, in the spleens of infected mice, large nests of Gr-1[dull] cells could also readily be observed throughout the red pulp (Figure 1D).

Previous reports have suggested monocytes, macrophages, and neutrophils as potent cellular sources of proangiogenic MMP-9 during inflammation.[19] In the present study, however, IHC analysis, *in situ* zymography, and flow cytometry all revealed that in both naïve mice and infected mice MMP-9 was colocalized almost exclusively with Gr-1^{bright} cells, with minimal MMP-9 expression in Gr-1^{int} monocytes/DC even after neutrophil depletion (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).

To determine the potential role of these two myeloid populations in regulating vascular remodeling during infection, we compared the effects of either depleting both populations (using Gr-1) or just Ly6G^{+} neutrophils (using 1A8). Neutrophil depletion was similar in mice treated with either mAb (Figure 1, E–H), whereas monocytes/DC were de-

pleted only after administration of Gr-1 (Figure 1, I and J). Immunohistology of 1A8-treated mice showed an almost complete absence of Gr-1^{bright} cells, a finding that, together with the flow analysis, helped to confirm that most Gr-1[dull] cells detected by this method were monocytes/DC (Figure 1K). Consistent with previous studies,[11] administration of Gr-1 mAb during established infection led to an increase in splenic parasite burden: 195 ± 17 versus 304 ± 40 Leishman-Donovan units (LDU) at day 28 after infection in isotype-treated versus Gr-1-treated mice, respectively ($n = 10$ mice; $P < 0.02$). Similarly, administration of 1A8 also increased parasite burden at day 28, from 53 ± 12 LDU to 168 ± 33 LDU ($n = 7$ mice; $P < 0.001$).

Ly6C[+] Cells are Selectively Involved in Red Pulp Vascular Remodeling

To assess the effects of monocyte and neutrophil depletion on splenic vascular remodeling, we performed quantitative IHC on spleen sections from Gr-1 and 1A8 treated
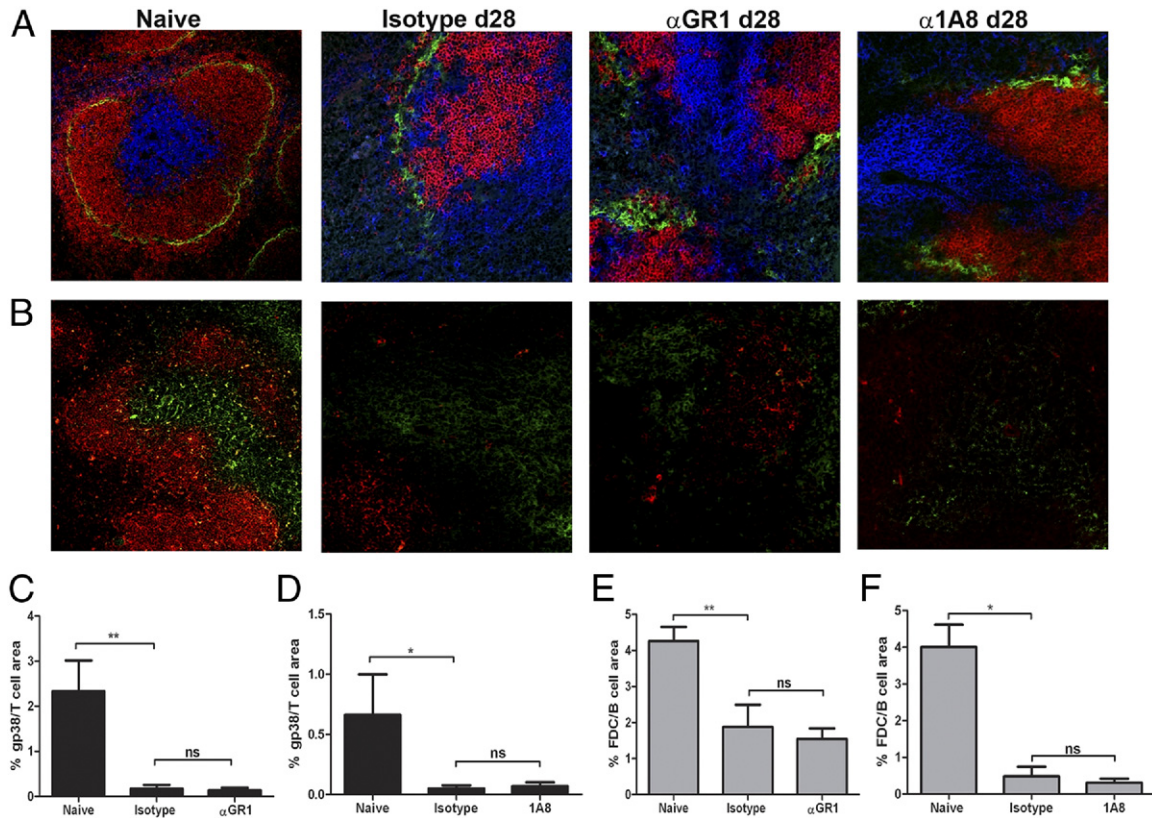
Figure 3. Neither Ly6C^{+} inflammatory monocytes/DC nor Ly6G^{+} neutrophils are responsible for white pulp remodeling. Spleens from *L. donovani* infected mice were removed at day 28 after infection and were analyzed, alongside spleens from naïve mice, by IHC. **A** and **B:** Spleen sections from naïve mice and infected mice treated with isotype control, Gr-1, or 1A8 from day 21 to day 28 of infection were stained to identify marginal metallophilic macrophages (CD169; green), B cells (B220; red), and T cells (CD3; blue) (**A**) and FRC (gp38, green) and FDC (FDCM1, red) (**B**). Original magnification, ×200. **C–F:** Computer-assisted quantitative morphometry was performed to determine the expression of gp38 (**C** and **D**) and FDCM1 (**E** and **F**) in mice treated with Gr-1 (**C** and **E**) and 1A8 (**D** and **F**). Data were generated from multiple sections examined from at least 5 mice per group. *$P < 0.05$, **$P < 0.01$.

mice (Figure 2). Meca-32 is a pan endothelial marker that recognizes red and white pulp vasculature and the endothelium of the marginal sinus in naïve mice.[6] As previously reported,[6] Meca-32^{+} vessels increased in abundance in the red pulp of mice infected with *L. donovani* (Figure 2A). Administration of Gr-1 mAb to mice from day 21 to day 28 after infection reduced the level of Meca-32 expression in red pulp to that seen in control uninfected mice (Figure 2, A and C). In the red pulp, some large-caliber vessels also stained for CD31 (data not shown), and the increase in CD31 in the red pulp observed in infected mice was also arrested after administration of Gr-1 mAb (Figure 2E). Strikingly, when Ly6G^{+} cells alone were depleted using 1A8, no effects were observed on either of these two measures of splenic red pulp vascular remodeling (Figure 2, A, D, and F). These findings indicate that vascular remodeling in the red pulp is likely to be dependent on the presence of inflammatory Ly6C^{+} monocytes/DC.

Neither Ly6C/G[+] nor Ly6G^{+} Cells Affect Vascular and Stromal Cell Remodeling in the White Pulp

Remodeling of the white pulp compartment also occurs during EVL.[6,20] In the white pulp, CD31 is expressed only

on the central arteriole in naïve mice, but with infection it is expressed also in the neovasculature that emerges during progressive infection.[6] Strikingly, neither administration of Gr-1 or 1A8 mAb had any effect on the neovascularization of the white pulp as measured by CD31^{+} vessels (Figure 2, B, G, and H).

Loss of the FDC and FRC stromal cell networks is also characteristic of EVL-induced lymphoid tissue remodeling and underpins a loss of T- and B-cell compartmentalization.[20] We also assessed, therefore, whether this process was regulated by either Ly6C/G[+] or Ly6G^{+} cells. Neither administration of Gr-1 nor 1A8 was able to reverse the disruption seen in T- and B-cell compartments (Figure 3A). Similarly, loss of FRC networks, measured using gp38 (Figure 3, B, C, and D), and loss of FDC networks, measured using FDCM1 (Figure 3, B, E, and F), was equivalent in both Gr-1 and 1A8-treated mice. Thus, by all criteria measured, white pulp remodeling was independent of the presence or absence of neutrophils and monocytes, consistent with the exclusion of these cells from the white pulp.

Discussion

The present findings provide a number of new insights into the pathogenesis of EVL-associated splenomegaly.

Most importantly, we show that Ly6C$^+$ inflammatory monocytes/DC located in the red pulp play a major role in remodeling the red pulp vasculature during ongoing splenomegaly. The observation that neither neovascularistion of the white pulp nor white pulp stromal cell networks were affected by monocyte/DC (or neutrophil) depletion suggests strongly that these cells can modify only their immediate environment.

Our data derived from this model of infection-induced splenic vascular remodeling are in contrast to a number of recent reports that implicated neutrophils as major agents of vascular remodeling: i) neutrophil-derived MMP-9 was able to direct angiogenesis in collagen onplants grafted onto the chorioallantoic membrane of chick embryos,[21] ii) neutrophil depletion caused a significant attenuation of microvessel growth in a model of brain focal angiogenesis induced by AAV-VEGF gene transfer,[22] and iii) neutrophil depletion reduced the number of angiogenic islet dysplasias in RIP1-Tag2 mice.[23] However, Gr-1 has often been used as a tool for neutrophil depletion *in vivo* and, as the present findings clearly indicate, the partial loss of monocytes that occurs as a result of Gr-1 administration can have profound functional consequences.

The present findings add to a growing body of evidence that implicates Ly6C$^+$CD11b$^+$ monocytes/DC/myeloid suppressor cells as regulators of vascular growth and remodeling. For example, such cells are closely associated with the vasculature found in spontaneous prostate cancers in TRAMP mice and produce a range of proangiogenic factors, including MMP-9.[24] Gr-1^{dull}CD11b$^+$ cells have been implicated in ischemia-associated neovascularization, with adoptive transfer of these cells improving local blood flow in mice with femoral artery dissection.[25] CD11b$^+$ macrophages have also been implicated in inflammation-induced angiogenesis and lymphangiogenesis in a model of cutaneous leishmaniasis, based on *in vivo* depletion studies using clodronate liposomes.[16] Even taking into account variations in phenotypic analysis, differences in methods of *in vivo* depletion, and different endpoints for scoring vascular remodeling, it is difficult to reconcile these data with a single angiogenic stage of monocyte/macrophage/DC differentiation. Rather, it seems likely that the potential for vascular remodeling exists within mononuclear phagocytes at multiple stages of their differentiation, influenced by local environmental factors.

In both macrophages and neutrophils, MMPs (and MMP-9 in particular) have been implicated as proangiogenic factors,[13,21,24] and human monocytes can be stimulated to produce this metalloproteinase by either alternative IL-4-mediated activation or IL-10-directed deactivation.[26] Both cytokines are readily detectable during EVL.[27] Taken together, these considerations led us to study the expression of MMP-9 in the spleens of *L. donovani* infected mice. Surprisingly, we were unable to demonstrate MMP-9 expression in any splenic mononuclear phagocyte population, whether by flow cytometry, IHC, or gelatinolytic assays. In neutrophils, however, MMP-9 was abundant. The absence of detectable levels of MM9 in splenic macrophages of mice infected with *L. donovani* would be consistent with the low levels of alternative activation seen in *L. donovani* infection,[28] but may also reflect tissue and/or species-specific differences in MMP-9 expression by macrophages. For example, F4/80$^+$ peritoneal macrophages expressed significantly more MMP-9 than did neutrophils during the early stages of zymosan-induced peritonitis,[29] consistent with a recent report that peritoneal macrophages infected *in vitro* with *L. chagasi* and cocultured with hepatocytes secrete elevated levels of active MMP-9.[30] Similarly, whereas in that study resident peritoneal neutrophils did not express detectable MMP-9,[29] in the present study neutrophils represent the only detectable MMP-9$^+$ population in the naïve spleen. Although the apparently tight regulation of MMP-9 expression during EVL is of interest, in the present context it appears that MMP-9 is unlikely to play a major role in red pulp vascular remodeling.

In summary, we have shown that splenic vascular remodeling during EVL takes place in a compartment-specific manner, with red pulp remodeling being regulated directly or indirectly by Ly6C$^+$ inflammatory monocytes recruited to this site during infection. Although our findings provide an important mechanistic insight into the vascularization process that accompanies splenomegaly, the compartment-specific activity of these monocytes also indicates that some disparate mechanism or mechanisms must be involved in neovascularization of the white pulp.[6] Further studies are now required to define the cells and factors involved in the latter process.

Acknowledgment

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