Loss of TNF Signaling Facilitates the Development of a Novel Ly-6C<sup>low</sup> Macrophage Population Permissive for *Leishmania major* Infection

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Loss of TNF Signaling Facilitates the Development of a Novel Ly-6C\textsuperscript{low} Macrophage Population Permissive for Leishmania major Infection

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In the absence of TNF, the normally resistant C57BL/6 (B6.WT) strain develops a fatal, progressive form of leishmaniasis after infection with Leishmania major. It is not yet understood which TNF activity or the lack thereof is responsible for the dramatic progression of leishmaniasis in TNF-negative (B6.TNF\textsuperscript{−/−}) mice. To elucidate the underlying mechanisms resulting in the fatal outcome of L. major infection in this gene-deficient mouse strain, we analyzed the monocytic component of the inflammatory infiltrate in the draining popliteal lymph node and the site of the infection using multicolor flow cytometry. The leukocytic infiltrate within the draining lymph node and footpad of B6.TNF\textsuperscript{−/−} mice resembled that of B6.WT mice over the first 2 wk of cutaneous L. major infection. Thereafter, the B6.TNF\textsuperscript{−/−} mice showed an increase of CD11c\textsuperscript{+}Ly-6C\textsuperscript{low}CCR2\textsuperscript{low} monocytic dendritic cells within the popliteal lymph node in comparison with B6.WT mice. This increase of inflammatory dendritic cells was paired with the accumulation of a novel CD11b\textsuperscript{+}Ly-6C\textsuperscript{low}CCR2\textsuperscript{low} population that was not present in B6.WT mice. This B6.TNF\textsuperscript{−/−} and B6.TNFR1\textsuperscript{−/−}-specific cell population was CD11b\textsuperscript{+}Ly-6G\textsuperscript{−}iNOS\textsuperscript{−}, not apoptotic, and harbored large numbers of parasites. The Journal of Immunology, 2012, 188: 000–000.

The intracellular protozoan parasite Leishmania spp. is an important human pathogen that infects significant numbers of people in the developing world and increasing numbers in industrialized countries (1). Infection with Leishmania spp. results naturally from the transmission of metacyclic procyclics by the sandfly host Phlebotomus spp. during a blood meal (2, 3) and can cause a spectrum of diseases ranging from the relatively benign cutaneous leishmaniasis to visceral leishmaniasis, which is fatal if left untreated (1, 4).

The s.c. infection of the mouse with Leishmania major, experimental cutaneous leishmaniasis, has provided a useful tool to study the role of cytokines in response to infection and to understand the genetic basis of the dichotomy of T cell subpopulations due to the expression of different cytokines. A large body of evidence has established that the protective immune response in mouse strains resistant to L. major infection, such as B6.WT, is dependent on the production of IFN-γ by CD4\textsuperscript{T} T cells (5, 6) and conferred by the action of the central effector molecule NO (7, 8). In contrast, infection of a susceptible mouse strain results in an early IL-4 burst (9) that counters the effects of IFN-γ and leads to progressive and ultimately lethal leishmaniasis (10).

A role for TNF in promoting resistance to intracellular parasitic pathogens such as L. major has been investigated in a range of experimental models (11), but the mechanisms by which this cytokine exerts its protective effects remain undefined. TNF is a pleiotropic cytokine that exists as both a soluble and a transmembrane homotrimer (12, 13). It shares homology with the related cytokine lymphotoxin α and binds to two cognate receptors, TNFR1 (p55, 60) and TNFR2 (p75, 80) (14, 15). It has been identified in vitro as a strong costimulatory signal that facilitates the sustained induction of the inducible NO synthase (iNOS) promoter in murine bone marrow (BM) macrophages (16–20). Although TNF is produced by different cell types, including CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (21) and macrophages (22, 23), albeit under distinct stimulatory requirements, several populations of inflammatory cells of monocytic origin have been identified recently as a major source of TNF in vivo after challenge with a range of pathogens such as Listeria monocytogenes or L. major. These cells belong to identical or similar subpopulations, termed TNF/iNOS-producing dendritic cells (TIP-DCs) (22), inflammatory DCs (24), inflammatory monocytes (25), or monocyte-derived DCs (Mo-DCs) (26) and are characterized by their expression of CCR2 as well as Ly-6C (25, 27). The importance of these cells in the context of intracellular infections is highlighted by the loss of Mo-DCs in CCR2\textsuperscript{−/−} mice due to a defect of the CCR2-mediated emigration of monocytes from BM into the periphery (28). The consequences are increased disease burden and mortality and have been demonstrated in a variety of infection models including L. major (29).

Therefore, on the basis of earlier findings, we hypothesized that the actions of TNF in inducing differentiation and ultimately effector functions in innate myeloid cells recruited to the site of infection contributed to the susceptibility seen in mice lacking TNF. In the current study, we examined the role of inflammatory monocytes during experimental cutaneous leishmaniasis using mice that lack...
constituents of different TNF signaling pathways. In direct contrast to an earlier publication (24), our results clearly show that the absence of TNF does neither impair the differentiation of monocytes to Mo-DCs nor the recruitment of Mo-DCs to sites of inflammation. The analysis of the infiltrate in L. major-infected B6. TNF−/− mice revealed the presence of an additional yet undefined monocyte population that only accumulated in the draining lymph nodes in the absence of TNF. These cells were negative for iNOS and served as reservoir for L. major, potentially supporting the rapidly fatal L. major infection observed in TNF−/− mice.

Materials and Methods

Mice

The gene-targeted C57BL/6 mouse strains deficient for soluble and membrane TNF (B6.TNF−/−) or for soluble TNF only (B6.men.TNF−/−), respectively, were generated on a genetically pure C57BL/6 (B6.WT) background as described (30, 31). The B6.TNFFR1−/− and B6.TNFFR2−/− mice were obtained from The Jackson Laboratory and had been backcrossed >10 times or had been established on a C57BL/6 background, respectively (32). The screening procedure followed the protocols published, respectively (32). All of the animals and were kept in specific pathogen-free conditions at the Animal Research Facilities of the Comparative Genomics Centre (Townsville, Australia) or the Menzies Research Institute Tasmania (Hobart, Tasmania, Australia). All of the experiments followed protocols approved by the animal ethics committees of James Cook University (Townsville, Australia) or the University of Tasmania (Hobart, Australia). Mice 8–12 wk of age were used in all of the experiments.

Parasites and infection

The virulent L. major isolate MHOM/IL/81/1FE/BNI or the enhanced GFP-expressing, transfected L. major isolate MHOM/IL/80/Friedlin-eGFP (33) (a gift from Dr. Emanuela Handman, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) were maintained through serial passage in BALB/c mice in vivo and cultured in vitro in Novy-MacNeal-Nicolle blood agar slants in RPMI 1640 containing 10% newborn calf serum, penicillin/streptomycin, nonessential amino acids, and 10 mM HEPES (34), all supplied by Invitrogen (Sydney, Australia). In addition, the eGFP-transgenic L. major parasites were cultured in the presence of 10 μM hydrocortisone B (Invitrogen) to maintain eGFP expression. For infection, stationary-phase L. major promastigotes were used between passages 2 and 6, and 3 × 10^7 parasites were injected in a volume of 40 μl into one hind footpad. The infected mice were monitored daily, and the lesion size was measured twice weekly using a metric caliper.

Flow cytometry

Tissue of draining popliteal lymph nodes or footpad lesions was incubated with collagenase D (1 mg/ml; Roche Diagnostics Australia, Brisbane, Australia) and DNase I (100 U/ml; Sigma-Aldrich, Sydney, Australia), for 30 min at 37°C and disrupted by mechanical disruption between frosted glass slides, and single-cell suspensions were prepared. Cells were filtered through 60-μm nylon mesh or 40-μm cell strainers (BD Biosciences, Melbourne, Australia) to remove tissue debris. Before FACS staining, the cells were blocked with anti-CD16/32 (clone 2.4G2; eBioscience, San Diego, CA) containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) and stained, sections were rehydrated in PBS containing 1% BSA for 60 min followed by a blocking with anti-CD16/CD32 (BD Biosciences). Alternately, CD11b+ cells were isolated using an AutoMACS (Miltenyi Biotec, Sydney, Australia) and CD11b magnetic beads following the manufacturer’s protocol. After the addition of Abs to surface molecules, the cells were fixed using Fix/Perm buffer (BioLegend) and permeabilized with 0.5% saponin in PBS according to the manufacturer’s protocol. After intracellular staining, the cells were put onto a microscope slide, and a coverslip was placed on top.

Polyclonal IgG Abs against L. major (clone V121, MHOM/IL/67/Trieger II) were purified from rabbit serum using protein G-Sepharose 4B (Invitrogen). Different monocytic and DC populations have been shown to be rapidly fatal L. major infection observed in TNF−/− mice.

Draining popliteal lymph node sections were dissected and rapidly frozen in liquid nitrogen vapor and stored at −80°C. Sections of 10 μm in thickness were cut using a cryotome (Thermo Shandon), air-dried, and fixed in acetone at −20°C. Before being stained, sections were rehydrated in PBS containing 1% BSA for 60 min followed by Fc blocking with anti-CD16/CD32 (BD Biosciences). Alternatively, CD11b+ cells were isolated using an AutoMACS (Miltenyi Biotec, Sydney, Australia) and CD11b magnetic beads following the manufacturer’s protocol. After the addition of Abs to surface molecules, the cells were fixed using Fix/Perm buffer (BioLegend) and permeabilized with 0.5% saponin in PBS according to the manufacturer’s protocol. After intracellular staining, the cells were put onto a microscope slide, and a coverslip was placed on top.

Polyclonal IgG Abs against L. major (clone V121, MHOM/IL/67/Trieger II) were purified from rabbit serum using protein G-Sepharose 4B (Invitrogen), followed by labeling with Cy5 mono- and FITC-conjugated antibodies (Amersham Biosciences, Buckinghamshire, U.K.) as described by the manufacturer. Popliteal lymph node sections were stained using polyclonal rabbit anti-L. major Cy5, mouse anti-mouse iNOS-FITC (6/105NOS/NO type II; BD Biosciences), and rat anti-mouse CD11b-biotin (M1/70; BD Biosciences). Single, isolated CD11b+ cells were stained using rat anti-mouse CD11b-biotin, rat anti-mouse F4/80/Alexa Fluor 488 (BMB; eBioscience), and rabbit anti-L. major-Cy5. Secondary staining was performed using streptavidin-Alexa Fluor 546 (Invitrogen) before being mounted with Mowiol (Calbiochem, La Jolla, CA) containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma-Aldrich) to prevent fading and allowed to dry overnight. Sequential images were acquired using either a Zeiss LSM710 or a Zeiss LSM510 confocal microscope in channel mode.

Statistical analysis

Statistical analysis was performed using a nonparametric Mann–Whitney U test or a Kruskal–Wallis test with Dunn’s multiple comparison test to test for multiple hypotheses where appropriate. Mean values ± SEM are shown as indicated. Analyses were performed using Prism 5.0 for Macintosh (GraphPad Software, San Diego, CA). Each experimental group was compared with B6.WT controls. Statistical values of p < 0.05 were considered to be significant and were labeled with *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001, respectively.

Results

Production of iNOS is not affected in L. major-infected B6. TNF−/− mice

Different monocytic and DC populations have been shown to be essential producers of iNOS (24). In a plethora of in vitro experiments, TNF has been demonstrated to be a central inducer of iNOS. The situation in vivo is more complex, and the role of TNF in the induction of the enzyme iNOS within the draining popliteal lymph nodes of B6.WT and B6.TNF−/− mice. Mice of both genotypes showed clustered iNOS+ areas within the paracortex of the popliteal lymph node at day 21 postinfection closely associated with...
L. major amastigotes and CD11b+ cells (Fig. 1). Interestingly, B6. WT mice showed strong and distinct CD11b expression, whereas in the absence of TNF this CD11b expression was generally more diffuse. Furthermore, at this point in time in the course of the disease, we observed discrete clusters of large, unusual CD11b+ cells strongly colocalized to areas with iNOS production and only present in B6.TNF−/− mice (36).

Analysis of the role of TNF and its receptors for the differentiation of CD11b+Ly-6C+CCR2+ Mo-DCs and other monocytic cell populations in the draining lymph nodes

To understand how the absence of TNF contributed to changes in the monocytic compartment of the inflammatory infiltrate in the draining popliteal lymph node, we undertook a comprehensive flow cytometric analysis throughout the course of the infection. The investigation of major conventional DC subsets (CD11c−CD8−, CD11c+CD8+) and plasmacytoid DCs (CD11b+CD11c+Ly-6C+ B220+) did not reveal substantial differences between B6.WT and B6.TNF−/− mice over an observation period of 4 wk (P.D. Fromm and H. Köhler, unpublished observations). At the peak of the disease after 2 wk of infection, an investigation of iNOS-producing inflammatory CD11b+Ly-6C+CCR2+ Mo-DCs (37) confirmed strong recruitment of this population to both the footpad lesion (P.D. Fromm and H. Köhler, unpublished observations) and the draining lymph nodes in B6.WT mice (26) and in mice deficient for TNF and its receptors (Fig. 2A), with statistically significantly increased absolute numbers in all of the analyzed mouse strains postinfection (Fig. 2B). However, a multiphypothesis comparison of all four genotypes shows only a statistical difference between B6.WT and B6.TNF1−/− mice. In contrast, the absence of either TNF or TNFR1 resulted in a significant increase of the total numbers of a yet undefined CD11b+Ly-6C+CCR2low population (Fig. 2). This cell population was essentially absent from infected B6.WT, B6.TNF2−/−, or B6.mtnf6−/− mice, and its appearance could not be reconciled with a published monocytic population.

Phenotypic characterization of CD11b+Ly-6C+CCR2+ Mo-DCs and the unknown CD11b+Ly-6C+CCR2low cell populations

To further analyze both infiltrating CD11b+ monocyte populations during murine leishmaniasis, we characterized the phenotypes of both populations in all of the genotypes (Fig. 3A, R1 and R2) with regard to their forward/side scatter characteristics and their expression of activation and differentiation markers and annexin V (Fig. 3B–E). The CD11b+Ly-6C+CCR2+ Mo-DC (Fig. 3A R2) population resembled in all of the analyzed markers the already described inflammatory Mo-DCs, having a typical leukocyte morphology as represented in their forward and side scatter distributions (Fig. 3C, R2) as well as expressing both CD115 and CD86 (Fig. 3D, 3E). Both B6.WT and B6.TNF−/− or TNFR−/− Mo-DCs expressed high levels of MHC II and CD86, with B6. TNF−/− and TNFR1−/− Mo-DCs displaying slightly higher levels than either B6.WT or B6.TNF2−/− Mo-DCs (Fig. 3B, R2), indicating that a large proportion of these cells was activated and capable of stimulating T cell responses. At day 21 postinfection, both B6.WT and B6.TNF−/− CD11b+Ly-6C+CCR2+ Mo-DCs from draining lymph nodes histologically resembled monocytes (P.D. Fromm and H. Köhler, unpublished observations).

In comparison, the accumulating CD11b+Ly-6C+CCR2low cells were predominantly negative for the expression of MHC II and CD86 in all of the genotypes (Fig. 3B, R1), but although B6.WT and B6.TNF2−/− cells displayed a characteristically small morphology as determined by forward and side scatter, both B6.TNF−/− and B6.TNF1−/− had increased side scatter profiles, typical of more granular, activated cells (Fig. 3C, R1).

Further phenotypic analysis showed that the small number of CD11b+Ly-6C+CCR2low cells appearing in B6.WT and B6. TNF2−/− mice were fundamentally different from the CD11b+ Ly-6C+CCR2low cells that accumulate in B6.TNF−/− and B6.TNF1−/− mice. CD11b+Ly-6C+CCR2low cells from B6. WT mice expressed slightly reduced levels of CD115, which did not reach statistical significance when corrections were made for

![FIGURE 1. Clustering of CD11b+ cells with L. major parasites in the draining popliteal lymph node is associated strongly with the expression of iNOS. (A and B) The localization of L. major parasites was determined with respect to the expression of both CD11b and iNOS in B6.WT (A) and B6.TNF−/− mice (B) in the draining popliteal lymph nodes at day 21 postinfection. A merged image of single-channel confocal images of sections stained with anti-L major (red), anti-iNOS (green), and anti-CD11b (blue) is shown (original magnification ×10). The inset shows an enlarged view of iNOS clustered regions identified in the image. Scale bar, 200 μm (in the overall image) and 20 μm (in the inset).](http://www.jimmunol.org/content/3/1/21.f1)

![FIGURE 2. Comparison of the inflammatory infiltrate in the draining lymph nodes during the course of L. major infection in B6.WT, B6.TNF−/−, B6.TNF1−/−, B6.TNF2−/−, and B6.mtnf6−/− mice. (A) CD11b+Ly-6C+CCR2+ monocytes and CD11b+Ly-6C+ inflammatory Mo-DCs are recruited into draining popliteal lymph nodes during the course of L. major infection (days 7, 14, and 21). A dot blot representative for three experiments (n = 5–6 mice per experiment) is shown. (B) A summary of the absolute numbers of CD11b+Ly-6C+CCR2+ monocytes and inflammatory CD11b+Ly-6C+CCR2low Mo-DCs of B6.WT and B6.TNF−/− mice of one experiment at day 21 postinfection is shown. Every point represents one individual mouse (n = 5–6; *p ≤ 0.01, statistical significance tested using a Kruskal–Wallis test with Dunn’s multiple comparison test).](http://www.jimmunol.org/content/3/1/21.f2)
multihypothesis testing (Fig. 3D, 3E). In addition, a large proportion of these CD11b+Ly-6ClowCCR2low monocytes in the draining lymph nodes of B6.WT, B6.TNF−/−, B6.TNFR1−/−, and B6.TNFR2−/− mice. (A) A representative dot plot characterizing the infiltrating myeloid cells in B6.WT, B6.TNF−/−, B6.TNFR1−/−, and B6. TNFR2−/− mice is shown. (B-D) The CD11b+Ly-6ClowCCR2low monocytic cells (R1) and the inflammatory CD11b+Ly-6C+CCR2+ Mo-DCs (R2) are analyzed regarding their activation (MHC class II and CD86) (B), their scatter morphology (forward/side scatter) (C), and their median fluorescent intensity (MFI) of CD115, Ly-6G, CD62L, and annexin V (D). (E) The MFI of CD115, Ly-6G, CD62L, and annexin V is summarized for all four genotypes. In the case of Ly-6G, only the MFI of the positive peak has been used for the quantitative analysis. (n = 6–8 per genotype; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, statistical significance tested using a Kruskal–Wallis test with Dunn’s multiple comparison test).

FIGURE 3. Phenotypic characterization of CD11b+Ly-6ClowCCR2low monocytic cells and inflammatory CD11b+Ly-6C+CCR2+ Mo-DCs in the draining lymph nodes of B6.WT, B6.TNF−/−, B6.TNFR1−/−, and B6.TNFR2−/− mice. (A) A representative dot plot characterizing the infiltrating myeloid cells in B6.WT, B6.TNF−/−, B6.TNFR1−/−, and B6. TNFR2−/− mice is shown. (B-D) The CD11b+Ly-6ClowCCR2low monocytic cells (R1) and the inflammatory CD11b+Ly-6C+CCR2+ Mo-DCs (R2) are analyzed regarding their activation (MHC class II and CD86) (B), their scatter morphology (forward/side scatter) (C), and their median fluorescent intensity (MFI) of CD115, Ly-6G, CD62L, and annexin V (D). (E) The MFI of CD115, Ly-6G, CD62L, and annexin V is summarized for all four genotypes. In the case of Ly-6G, only the MFI of the positive peak has been used for the quantitative analysis. (n = 6–8 per genotype; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, statistical significance tested using a Kruskal–Wallis test with Dunn’s multiple comparison test).

increased levels of cell surface phosphatidylserine as measured by annexin V staining (p ≤ 0.001 and p ≤ 0.05, respectively), which is typical of apoptosis and consistent with the short half life of neutrophils (39, 40) and supports that they are fundamentally different from the accumulating cells observed in B6.TNF and B6. TNFR1−/− mice. These observations show that in the absence of intact TNFR1 signaling the recruitment of Mo-DCs to the inflamed lymph node is not impaired but rather induces the accumulation of a novel monocytic CD11b+Ly-6ClowCCR2low subset that is distinct from that observed in resistant B6.WT or B6. TNFR2−/− mice and, in fact, from any other described cell population.

The CD11b+Ly-6ClowCCR2low population harbors L. major and does not produce NO
ascertain in vivo the localization of the parasite together with IFN-γ-dependent effector functions such as NO production in popliteal lymph node-infiltrating Mo-DCs.

Examination of the draining lymph nodes at day 21 postinfection revealed the accumulation of two distinct CD11b+ myeloid populations in B6.TNF$^{-/-}$ mice that differed with respect to both iNOS expression and the presence of eGFP$^+$ L. major amastigotes (Fig. 4) and correlated with the two distinct CD11b$^+$ myeloid cell populations observed previously in B6.TNF$^{-/-}$ and B6.TNF$^{+/+}$ infected mice. The population in R1 (Fig. 4A) corresponded to the CD11b$^+$Ly-6C$^{low}$CCR2$^{low}$ population, whereas gate R2 was equivalent to CD11b$^+$Ly-6C$^+$CCR2$^+$ Mo-DCs (Figs. 2, 3). Analysis of eGFP expression as a surrogate of the presence of L. major amastigotes revealed that the majority of cells within gate R1 in B6.WT mice were uninfected (Fig. 4B). In contrast, the majority of the cells present within the same gate in B6.TNF$^{-/-}$ mice at day 21 harbored high parasite loads. Interestingly, despite the large number of L. major-harboring cells present in R1 in infected B6.TNF$^{-/-}$ mice, the total number of uninfected (L. major-eGFP$^+$) cells remained largely unchanged in both genotypes (Fig. 4A, lower left quadrant). Further analysis showed that these uninfected cells present in both B6.WT and B6.TNF$^{-/-}$ mice expressed the granulocyte-specific surface marker Ly-6G and were phenotypically similar in both genotypes (P.D. Fromm and H. Körner, unpublished observations), thus supporting our previous observation (Fig. 3). This stands in contrast to the L. major-eGFP$^+$CD11b$^+$Ly-6C$^{low}$CCR2$^{low}$ cells in B6.TNF$^{-/-}$ mice (Fig. 4A, R1, lower right quadrant) that display the altered phenotype described previously. Conversely, CD11b$^+$Ly-6C$^+$CCR2$^+$ Mo-DCs (Fig. 3, R2) from both B6.WT and B6.TNF$^{-/-}$ mice displayed the L. major-eGFP signal but showed concurrent iNOS expression (Fig. 4B). Quantitative analysis of the popliteal lymph nodes revealed an overall increase in total number of iNOS$^+$ cells in the lymph nodes of B6.TNF$^{-/-}$ mice that corresponded with the overall increase in the numbers of CD11b$^+$Ly-6C$^+$CCR2$^+$ Mo-DCs described previously (Fig. 4B, compare with Fig. 2). Further quantification indicated a significant increase in the number of CD11b$^+$Ly-6C$^{low}$CCR2$^{low}$ cells in B6.TNF$^{-/-}$ mice (Fig. 4B). However, as a caveat, it should be added that our flow cytometric analysis could underestimate the contribution of these highly infected cells to the total burden of infection, because we cannot exclude the possibility that these cells are sensitive to changes in pressure during the analysis and their number therefore might be underreported.

A subsequent microscopic analysis of CD11b$^+$ cells isolated from B6.WT and B6.TNF$^{-/-}$ mice confirmed the presence of L. major Ag within the cells in $>$50% of the observed cells from

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**FIGURE 4.** Characterization of iNOS expression in CD11b$^+$CCR2$^+$ and CD11b$^+$CCR2$^{low}$ cells from B6.WT and B6.TNF$^{-/-}$ mice infected with fluorescent L. major-eGFP. (A) B6.WT and B6.TNF$^{-/-}$ mice were infected with L. major-eGFP promastigotes, and draining popliteal lymph nodes were harvested at day 21 postinfection. CD11b$^+$Ly-6C$^{low}$CCR2$^{low}$ monocytic cells (R1) and CD11b$^+$Ly-6C$^+$CCR2$^+$ Mo-DCs (R2) were analyzed by flow cytometry for intracellular iNOS and eGFP expression. (B) The absolute numbers of total iNOS$^+$ cells and of CD11b$^+$CCR2$^+$ and CD11b$^+$CCR2$^{low}$ inflammatory cells are summarized (n = 5; *p ≤ 0.05, **p ≤ 0.01). Statistical significance was tested using a Mann–Whitney U test. One of two independent experiments is shown. (C) Comparison of the burden of infection of B6.WT and B6.TNF$^{-/-}$CD11b$^+$ monocytes. CD11b$^+$ monocytes were isolated using magnetic beads and stained with anti-CD11b, anti-F4/80 mAb, and anti-L. major serum. Original magnification ×630.
B6.TNF<sup>−/−</sup> mice, whereas we were unable to find any infected B6.WT cells (Fig. 4C).

This indicates that the lack of TNF does not impact directly on the iNOS potential of Mo-DCs infiltrating the draining lymph node in response to infection with <i>L. major</i>. However, it reveals a role for TNF in promoting a susceptible phenotype in other infiltrating cells, which results in the creation of a niche for the parasites that is removed from the effector mechanisms needed to facilitate parasite clearance.

**Development of CD11b<sup>+</sup>Ly-6C<sup>+</sup>CCR2<sup>+</sup> Mo-DCs and the unknown CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> cells after <i>L. major</i> infection is not dependent on intrinsic responsiveness to TNF signaling**

Because TNF has been shown to influence DC and macrophage development and activation (41, 42), the unique CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> cell population that we observe in the absence of TNF may result from either alterations in monocyte development or accumulation of parasite-infected cells due to a failure of these cells to die postinfection in the absence of TNF. To test how the differentiation of CD11b<sup>+</sup>Ly-6C<sup>+</sup>CCR2<sup>+</sup> Mo-DCs and the unknown CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> monocytes after <i>L. major</i> infection is correlated with either TNFR and consequently analyze the intrinsic responsiveness of infiltrating myeloid cells to TNF, we generated mixed BM chimeras utilizing B6.WT, B6.TNFR1<sup>−/−</sup>, and B6.TNFR2<sup>−/−</sup> cells in two different three-way combinations, exploiting the congenic loci available for CD45 and CD90. This allowed us to examine the individual contributions of TNFR1 or TNFR2 signaling to the development of the monocytic infiltrate in footpad and draining lymph node of chimeric mice displaying equal levels of BM reconstitution (P.D. Fromm and H. Körner, unpublished observations) at day 14 after <i>L. major</i> infection.

In the draining popliteal lymph nodes, both the WT/WT and WT/TNFR2<sup>−/−</sup> donor combinations developed a strong CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> Mo-DC infiltrate, which in both groups was derived equally from both donor genotypes (Fig. 5A). Consistent with our previous findings, the CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> cell population (R1; Figs. 3, 4) was hardly detectable in either WT/WT or WT/TNFR2<sup>−/−</sup> chimeras (Fig. 5A). In contrast, the WT/TNFR1<sup>−/−</sup> mixed chimeras contained both CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> inflammatory Mo-DCs and CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> cells within the draining lymph node (Fig. 5A), similar to what we had described earlier in infected B6.TNF<sup>−/−</sup> or B6.TNFR1<sup>−/−</sup> mice (compare with Fig. 2). Interestingly, in this experimental approach, both Mo-DC and <i>L. major</i>-harboring CCR2<sup>low</sup>Ly-6C<sup>low</sup> cell populations also could be shown to originate from both WT (CD45.1<sup>+</sup>) and TNFR1<sup>−/−</sup> (CD45.2<sup>+</sup>) BM progenitor cells (Fig. 5A; p < 0.01) but not TNFR2<sup>−/−</sup> (p > 0.05). These data highlight that the differences in both CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>+</sup> Mo-DCs and CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> cells occurred independently of their individual responsiveness to autocrine or paracrine TNF. Analysis of the footpad lesion showed similarities between WT/WT, WT/TNFR1<sup>−/−</sup>, and WT/TNFR2<sup>−/−</sup> mixed chimeras with a strong influx of both CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>+</sup> Mo-DCs and CD11b<sup>+</sup>Ly-6C<sup>low</sup>CCR2<sup>low</sup> cells (Fig. 5B). However, unlike the Ly-6C<sup>low</sup>CCR2<sup>low</sup> myeloid cells in the draining lymph node, the cells that accumulated in the footpad were phenotypically similar and expressed high levels of Ly-6G, making them more akin to a neutrophilic infiltrate than a monocytic infiltrate (P.D. Fromm and H. Körner, unpublished observations).

**Discussion**

A central role for TNF and its receptors in mediating resistance to intracellular infection such as experimental cutaneous leishmaniasis is well established (36, 43, 44). In the absence of soluble TNF or TNFR1, the response to <i>L. major</i> is impaired severely and results in progressive and maintained infection that coincides with visceralization of the parasite and ultimately leads to a fatal outcome (36). The mechanisms that contribute to the lack of resistance in the absence of TNF are still not understood. We show in this investi...
gation that differentiation and functions of Mo-DCs such as iNOS production are not significantly TNF dependent. Furthermore, we demonstrate in the absence of the TNF/TNFRI signaling pathway a significant infiltration of Ly-6C<sup>+</sup>CCR<sup>+</sup> Mo-DCs and the accumulation of a yet undefined monocytic cell population of Ly-6C<sup>low</sup> CCR<sup>2</sup>low mononuclear cells. We hypothesize that this new monocytic cell population serves as a niche for parasites outside the reach of the innate immune response and contributes to the rapidly fatal course of *L. major* infection in TNF<sup>−/−</sup> mice.

Monocytes have long been considered to be a circulatory precursor population for tissue-resident and inflammatory macrophages. Through the use of adoptive cell transfers, two monocyte populations with different expression patterns of two chemokine receptors were demonstrated to coexist in the periphery (25). These chemokine receptors were used to define the functions of these populations. Monocytes of the Cx<sub>3</sub>,CR<sub>1</sub><sup>low</sup>Ly-6C<sup>−</sup>CCR<sup>2</sup>low subset were recruited to inflammatory sites apparently by TNF-independent mechanisms and relied primarily on the main ligand CCL2 for migration to inflamed peripheral tissues (45). The Cx<sub>3</sub>,CR<sub>1</sub><sup>high</sup>Ly-6C<sup>−</sup>CCR<sup>2</sup>high<sup>low</sup> subset was characterized by patrolling the luminal side of vessels in noninflamed tissues (46). The inflammatory Ly-6C<sup>−</sup>CCR<sup>−</sup> cell population described in this initial study was shown to differentiate into a variety of macrophage and Mo-DC subsets under inflammatory conditions, whereas the Cx<sub>3</sub>,CR<sub>1</sub><sup>high</sup>Ly-6C<sup>−</sup>CCR<sup>2</sup>high<sup>high</sup> monocytes appeared to serve more homeostatic or regulatory functions (47). Recent investigations have expanded the role of these cells and have demonstrated that they also can develop into DCs, plasmacytoid DCs, and monocytes, including peripheral monocytes (48). One subset of Mo-DCs, originally described in the *L. monocytogenes* model and referred to as TIP-DCs (22), presented with strong secretion of NO (49). Meanwhile, similar cells have been described in different tissues and in infection models such as listeriosis (22), toxoplasmosis (50, 51), and trypanosomiasis (52).

Interestingly, especially infection with intracellular parasites such as *L. monocytogenes* or *Toxoplasma gondii* seems to result in the accumulation of those iNOS- and TNF-producing CCR<sup>2</sup> TIP-DCs in the spleens or peritoneum of infected mice (22, 50, 51). Infection with *L. major* also results in strong recruitment of TNF/iNOS-producing Mo-DCs to the lesion and draining lymph nodes (24, 26). Both Ly-6C<sup>−</sup>CCR<sup>−</sup> inflammatory monocytes as well as iNOS<sup>+</sup> cells are present in statistically increased numbers in TNF<sup>−/−</sup> mice as compared with infected B6.WT mice at day 21 postinfection, which represents the peak of acute disease. This is in contrast to recently reported findings that show a marked reduction in the numbers of CD11b<sup>+</sup>CD11c<sup>+</sup>iNOS<sup>+</sup> cells in TNF<sup>−/−</sup> mice of a mixed genetic background at day 28 postinfection (24). This obvious discrepancy could be due to the later time point at which the tissue was analyzed, the use of a mixed genetic background of the host mouse strain (11), or the use of a transgenic *L. major* (Friedlin) strain in contrast to the highly infective BNI granular side scatter characteristics. Although we cannot rely entirely on the quantification of the contribution of these cells to the inflammatory infiltrate because an undetermined proportion of these highly infected cells could rupture easily during flow cytometric analysis, we can be certain that these cells constitute a significant population in this infection. The reason for the accumulation of these highly parasitized cells could reflect defects that are connected directly to the absence of the TNF/TNFRI signaling pathway, such as the impaired induction of iNOS, which is correlated with reduced antileishmanial capacity as shown in earlier *L. major* infection experiments (53). Alternative explanations could be a skewed differentiation of monocytes in TNF<sup>−/−</sup> or TNFR<sup>1</sup>−/− mice leading to the generation of suppressive monocyte subsets or a parasite host interaction that is normally overcome in the presence of TNF. Interestingly, the phenotype of these cells resembles either alternatively activated macrophages (M2), which are generated in the presence of CD40<sup>+</sup> T cells, GM-CSF, and IFN-γ and have a role in healing (54), or a subset of myeloid-derived suppressor cells (55), which have been shown to be present in leishmaniasis (56) or, most likely, represent the accumulating CX<sub>3</sub>,CR<sub>1</sub><sup>high</sup> population that hypothetically would develop under normal circumstances in the presence of TNF to inflammatory monocytes but remain in our model in an earlier developmental stage, thus indicating a new role for TNF.

Evaluation of WT/TNFRI<sup>−/−</sup> mixed BM chimeras during infection with *L. major* allowed us to evaluate the specific intrinsic contributions of the two TNF signaling pathways to the development of the inflammatory monocyte compartment from genetically distinct BM progenitors. Interestingly, in our mixed BM chimeras, these Ly-6C<sup>low</sup>CCR<sup>2</sup>low cells, which were associated with a susceptible phenotype, arose only from WT and TNFR<sup>1</sup>−/− but not WT and TNFR2<sup>−/−</sup> BM progenitors. Therefore, the intrinsic role for TNF in controlling the infectivity of *L. major* has to be questioned because the accumulation of parasitized Ly-6C<sup>low</sup>CCR<sup>2</sup>low myeloid cells from both B6.WT- and B6.TNFRI<sup>1</sup>−/−-derived BM progenitors would argue for TNF needed to act in trans to promote an effective leishmanicidal innate effector response. This is supported by our data that show that memTNF<sup>ΔΔ</sup> mice do not display an accumulation of cells with a Ly-6C<sup>low</sup>CCR<sup>2</sup>low phenotype (Fig. 2). A role for membrane TNF on myeloid BM progenitors in providing protection to intracellular infection with *M. tuberculosis* has been shown in reciprocal memTNF<sup>ΔΔ</sup>/TNF<sup>−/−</sup> BM chimeras in which hematopoietic cells derived from memTNF<sup>ΔΔ</sup> mice conveyed a protective phenotype to infection, whereas adoptive transfer of either WT or memTNF<sup>ΔΔ</sup> T cells did not (57). This also would be in agreement with our earlier observation that cells of hematopoietic origin need to be able to produce TNF to facilitate control of intracellular *L. major* parasites (36).

Taken together, our results demonstrate that TNF controls important parts of the innate response during *L. major* infection. A
new monocyte cell population could be detected in mice deficient for either TNF or TNFR1 but not TNFR2 or membrane TNF. These cells lacked iNOS and were highly infected but not apoptotic. In the lymph nodes of infected mixed BM chimeras, we could show that these cells originated from both the B6.WT and the B6.TNFRI components. This would argue for reverse signaling through membrane TNF.

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Disclosures
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