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Brief Conclusive Report

Myeloid-derived suppressor cells help protective immunity to *Leishmania major* infection despite suppressed T cell responses

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ABSTRACT

Th1/Th2 cytokines play a key role in immune responses to *Leishmania major* by controlling macrophage activation for NO production and parasite killing. MDSCs, including myeloid precursors and immature monocytes, produce NO and suppress T cell responses in tumor immunity. We hypothesized that NO-producing MDSCs could help immunity to *L. major* infection. Gr1^{hi}(Ly6C^{hi}) CD11b^{hi} MDSCs elicited by *L. major* infection suppressed polyclonal and antigen-specific T cell proliferation. Moreover, *L. major*-induced MDSCs killed intracellular parasites in a NO-dependent manner and reduced parasite burden in vivo. By contrast, treatment with ATRA, which induces MDSCs to differentiate into macrophages, increased development of lesions, parasite load, and T cell proliferation in draining LNs. Altogether, these results indicate that NO-producing MDSCs help protective immunity to *L. major* infection, despite suppressed T cell proliferation. *J. Leukoc. Biol.* 90: 1191–1197; 2011.

Introduction

MDSCs expressing Gr1 and CD11b (Mac-1) include Ly6C^{hi} monocytic and Ly6G^{hi} granulocytic BM precursors, which inhibit T cell proliferation and play a deleterious role in tumor immunity [1–3]. Among MDSCs, suppressor monocytes, bearing the Gr1^{hi}CD11b^{hi}F4/80^{int} phenotype, suppress T cell-mediated responses in a NO-dependent manner [4, 5]. Immature or inflammatory monocytes [6, 7] expressing Gr1^{hi}CD11b^{hi}F4/80^{int} and iNOS are essential for control of parasite replication in oral infection with *Toxoplasma gondii* [8]. Moreover, increased susceptibility to *T. gondii* infection correlates with defective recruitment of Gr1^{+} inflammatory monocytes in CCR2-deficient mice [8, 9]. Gr1^{hi}CD11b^{hi} MDSCs are also recruited in lung infection with *T. gondii* [10] and to spleens [11] and hearts [12] of mice infected with *Trypanosoma cruzi*. However, how suppressor versus effector activities of MDSCs affect immunity to protozoan parasites remains unknown.

During chronic *Leishmania major* infection, myeloid precursors circulate in the blood, and purified blood monocytes phagocytose *L. major* promastigotes [7]. In addition, Gr1^{hi} cells are recruited to draining LNs [13]. Furthermore, immature mononuclear cells infiltrate skin lesions in acute infection by *L. major* [14–16], where they may act as host and effector cells [17]. CCR2 KO mice, which are defective in mobilization of monocytes from BM [8], are more susceptible than WT mice to *L. major* infection [17–19]. Accordingly, WT but not CCR2 KO mice more efficiently control infection by transgenic *L. major* parasites expressing MCP-1 (CCL2) chemokine through increased recruitment of CCR2^{+} monocytes and parasite killing [20]. How immature monocytes as host and suppressor cells affect immunity to *Leishmania* is still poorly understood.

Immature cells expressing myeloid cell markers but not F4/80 predominate as parasite reservoirs in skin lesions of BALB/c mice, even 4 weeks postinfection with *L. major* [14, 15, 21, 22]. By contrast, myeloid precursors from BM of resistant B6 mice develop leishmanicidal activity, as they begin to express F4/80 [15, 16]. Kinetic differences in maturation of monocyte/macrophage lineage in *L. major* infection may underlie development of susceptible versus resistant phenotypes in mice [14–16]. When adaptive immunity ensues, resistant hosts develop a protective type 1 immune response mediated by IFN-γ-producing CD4 Th1 cells, which activate

Abbreviations: B6 mice=C57BL/6 mice, BM=bone marrow, CNPq=Conselho Nacional de Desenvolvimento Científico e Tecnológico, dpi=days postinfection, hi=high, int=intermediate, KO=knockout, L-NIL=L-N6-iminoethyl-lysine, Mac-1=macrophage-1 antigen, MDSC=myeloid-derived suppressor cell, neg=negative, PEC=peritoneal exudate cells, Treg=regulatory T cell, UFRJ=Federal University of Rio de Janeiro

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NO production by macrophages to control parasite infection [23–25]. Intact IFN-γR signaling in macrophages, monocytes, and DCs is required for immunity to *L. major* infection [26]. By contrast, type 2 cytokines, such as IL-4 and IL-10, secreted by CD4 Th2 and Tregs, inhibit macrophage activation and may contribute to parasite persistence in susceptible hosts [23–25, 27, 28].

We investigated how MDSCs affect immunity to *L. major*. Here, we show that $Gr1^{hi}(Ly6C^{hi})CD11b^{hi}F4/80^{int}$ MDSCs induced upon acute *L. major* infection kill intracellular *L. major* parasites in a NO-dependent manner and reduce parasite burden in vivo. Furthermore, treatment with ATRA, which induces MDSCs to differentiate into mature macrophages [29], increased development of lesions and parasite load upon *L. major* infection. Paradoxically, NO-producing MDSCs suppress T cell responses but help immunity to *L. major* infection as effector cells.

MATERIALS AND METHODS

Animals and *L. major* infection

Female B6 mice were obtained from the UFRJ and Oswaldo Cruz Foundation (FIOCRUZ; Rio de Janeiro, Brazil). All animal experiments were approved by and conducted in accordance with guidelines of the Ethics Committee for Use of Animals (UFRJ). *L. major* LV39 parasites were cultured until stationary phase in Schneider's medium (Invitrogen Life Technologies, Carlsbad, CA, USA). Mice, aging 7–9 weeks, were infected in hind footpads with s.c. injection of 3×10^6 parasites/30 μl. In some experiments, mice were infected in both footpads and injected with ATRA (Sigma-Aldrich, St. Louis, MO, USA) or control vehicle. Infected mice received 30 μl ATRA (10 μM) in the left footpad and DMSO (0.02%) in the right footpad, 1 day and 8 days after infection. Lesions, measured with a vernier caliper (Mitutoyo America, Aurora, IL, USA), were expressed as the difference (Δ) between the left (ATRA) and the right (control) footpads.

Parasite load

Popliteal LNs were cultured in Schneider's medium, supplemented with 2 mM glutamine and 2% human urine, plus 10% FBS at 28°C for 72 h. Parasite loads were determined by serial dilution assay, and viable promastigotes were counted on a Neubauer chamber [30, 31].

Flow cytometry and cell sorting

B6 mice were injected in the peritoneum with *L. major* (3×10^6/200 μl) or PBS. PECs were collected after 24 h, washed in FACS buffer (containing 2% FBS), and incubated with anti-CD16/CD32 for Fc blocking, followed by staining with allophycocyanin-, PE-, PE-Cy7-, PerCP-Cy5.5-, or FITC-labeled anti-CD11b/Mac-1 and anti-Gr1, -Ly6C, -Ly6G, or -F4/80 [BD PharMingen (San Diego, CA, USA); except the anti-F4/80 mAb from Caltag (S. San Francisco, CA, USA) or eBioscience (San Diego, CA, USA) and anti-Ly6C from BD PharMingen or eBioscience]. For detection of NO, cells were first incubated with 5 μM of the DAF-diacetate probe (Molecular Probes, Invitrogen Life Technologies) for 15 min. Cells were acquired on a FACSCalibur system by using CellQuest software (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). PECs from mice injected with *L. major* were stained with allophycocyanin-, PE-, and FITC-labeled mAb to Gr1 (Ly6C/G, RB6-8C5), Mac-1/CD11b (M1/70), and F4/80 (BM8, eBioscience) and sorted in a MoFlo cytometer (Cytomation, Ft. Collins, CO, USA). We sorted cells with high expression of Gr1 and CD11b, which express intermediate levels of F4/80. $F4/80^{hi}$ macrophages and cells with high side-scattering were excluded [7]. Sorted cells were fixed, stained (Panotic staining kit, Laboclin, Brazil), and observed in an optical microscope (Eclipse E800, Nikon, Japan). Most (80–95%) of sorted cells expressed the $Gr1^{hi}CD11b^{hi}F4/80^{int}$ phenotype and a monocyte morphology (see Figs. 1C and 3A).

T cell culture

LN cells from mice infected with *L. major* (15 dpi) were resuspended in DMEM (Invitrogen Life Technologies), supplemented with 2 mM glutamine, 5×10^{-5} M 2-ME, 10 μg/ml gentamicin, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, and 10 mM HEPES (culture medium), plus 10% FBS (Invitrogen Life Technologies). For suppression of T cell proliferation, cells were cultured (1.5×10^5/well) in the presence or absence of sorted monocytes (1.5×10^5/well), stimulated during 48 h with plate-bound anti-CD3 (10 μg/ml) or *L. major* antigens (50 μg/ml frozen-thawed promastigotes), and treated with 0.5 mM L-NIL (Cayman Chemical, Ann Arbor, MI). Supernatants were collected for detection of nitrites [32] and IFN-γ by using a sandwich ELISA, according to the manufacturer's instructions (BD PharMingen). Cultures were pulsed with 1 μCi [H^3] thymidine for scintillation spectroscopy.

Parasite killing assays

IgG-labeled, sorted cells (5×10^4 cells/50 μl) were immobilized by panning in slides covered with mouse anti-rat IgG (10 μg/ml, MAR-18.5) during 1 h and infected with *L. major* parasites (five promastigotes/cell) for a further 3 h. Cells were washed and cultured in 0.5 ml culture medium plus 10% FBS, containing or not IFN-γ (2 ng/ml, R&D Systems, Minneapolis, MN, USA), IL-4 (5 ng/ml, R&D Systems), and L-NIL (1 mM) in 24-well vessels for 48 h. Slides were stained as above and evaluated for morphology and the presence of intracellular amastigotes within monocytes. Independent of treatment, 75% of cells retained monocytic morphology upon 48 h of culture, whereas the remaining 25% of cells differentiated into macrophages. *L. major* killing by monocytes was estimated as described previously [15]. For the effects of ATRA on monocyte differentiation and parasite killing, PECs (1.5×10^5/well) or sorted monocytes (1×10^5/well) were infected and cultured as above in the presence or absence of ATRA (2 μM). Slides were stained and evaluated for cell morphology and macrophage infection. For in vivo experiments, mice were infected with *L. major* (2×10^6 promastigotes/20 μl) in both footpads and injected within 3 h with sorted $Gr1^{hi}CD11b^{hi}$ cells (3.5×10^5 cells/20 μl) in the left footpad only.

Statistics

Results are expressed as average and SEM in figures. Data were first tested for normal distribution by using the Kolmogorov-Smirnov test and then analyzed by unpaired (independent analysis) or paired (repeated or dependent measures) Student's *t* test. For in vitro experiments, data are expressed as average of triplicates/treatment, and the number of experiments is indicated in figure legends.

RESULTS AND DISCUSSION

Previous studies [14] indicate that mononuclear cells comprise the majority (60–80%) of cells in cutaneous lesions of resistant B6 but not susceptible BALB/c (30–40%) mice in the first 2 weeks of *L. major* infection. Fifty percent to 80% of *L. major* parasites reside within monocytes or immature macrophages in lesions of both mouse strains [14]. Moreover, inflammatory monocytes expressing $Gr1^{hi}CD11b^{hi}F4/80^{int}$ are recruited early upon *L. major* infection in a CCR2-dependent manner and act as effector cells [17].

L. major infection elicits Gr1^{hi}(Ly6C^{hi})CD11b^{hi} MDSCs

To investigate whether infection with *L. major* elicits monocytes with features of MDSCs during the first 2 weeks of infection, B6 mice were injected with *L. major* in footpads, and cells from lesions were analyzed by flow cytometry. Cells found in footpad lesions upon 2 weeks of infection expressed Gr1^{hi}CD11b^{hi} and intermediate levels of F4/80 (**Fig. 1A**). In addition, B6 mice were injected with *L. major* in the peritoneum to obtain larger numbers of cells for

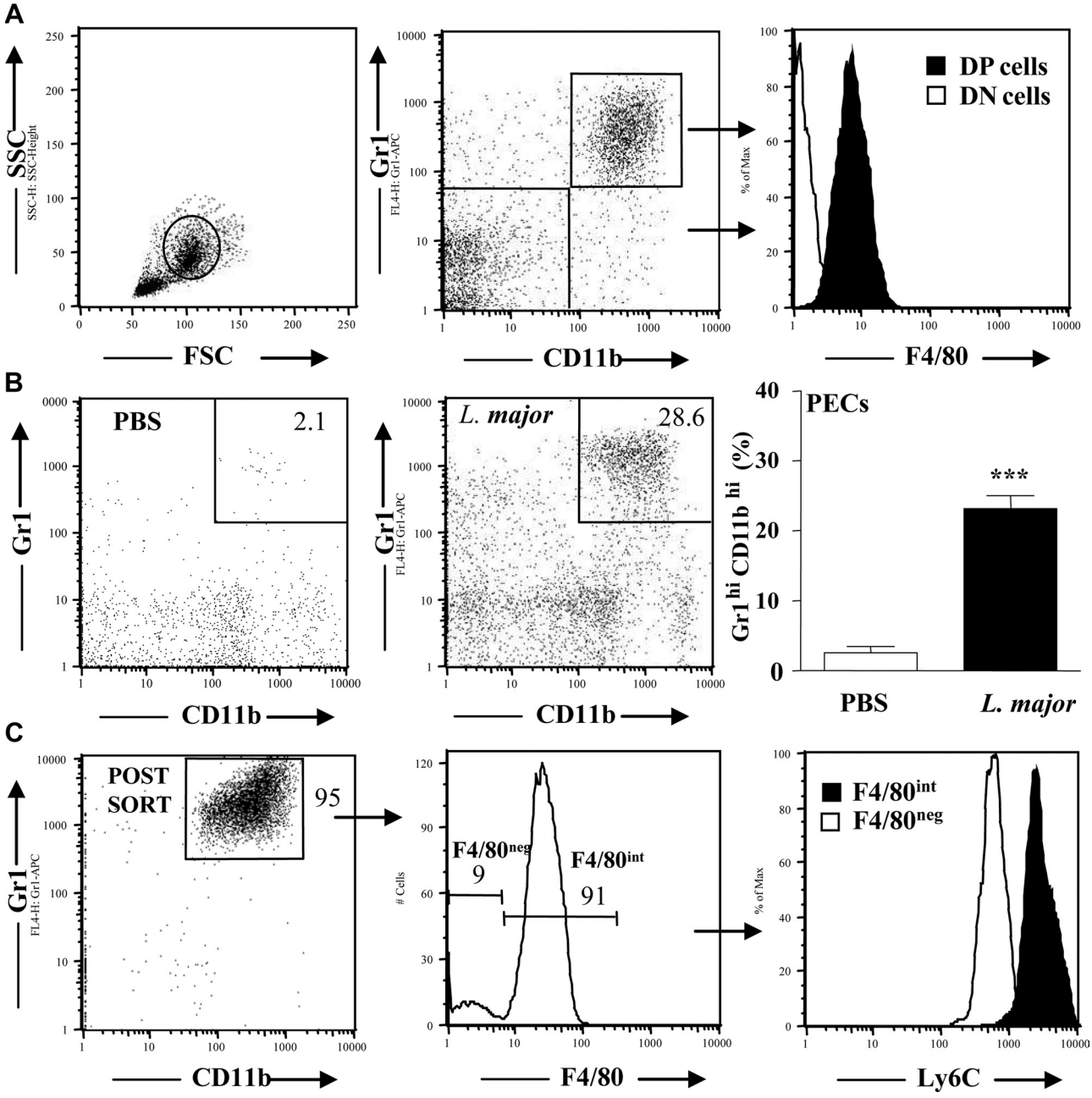

Figure 1. MDSCs recruited to local infection with *L. major*. (A) Dot plots represent cells with higher forward-scatter (FSC) in footpad lesions from 2 week-infected mice (circle). Total tissue cells were analyzed for Gr1 and CD11b expression (left and middle panels). Gr1^{hi}CD11b^{hi} [double-positive (DP)] cells from mice infected with *L. major* were analyzed further for F4/80 and compared with Gr1^{neg}CD11b^{neg} [double-negative (DN)] lymphocytes (right panel). SSC. Side-scatter. (B) PECs collected upon 24 h of i.p. injection with *L. major* or PBS in B6 mice. Left and middle panels show dot plots for CD11b and Gr1 expression and percentage of Gr1^{hi}CD11b^{hi} cells. A significant difference in the recruitment of cells between mice injected with PBS and *L. major* is indicated (***) for $P < 0.001$ (n=6 mice/group; right panel). (C) Middle and right panels show F4/80 and Ly6C expression in sorted (POST SORT) Gr1^{hi}CD11b^{hi} cells (left panel). Results represent at least three independent experiments.

analyses and cell sorter experiments. $Gr1^{hi}CD11b^{hi}$ cells were recruited to peritoneum 24 h upon i.p. injection of *L. major* but not PBS (Fig. 1B). Most gated $Gr1^{hi}CD11b^{hi}$ cells expressed $F4/80^{int}$ and NO (not shown). The phenotype of cells found in infected mice closely resembles that of immature/suppressor monocytes characterized previously as MDSCs in spleens, where $Gr1^{hi}CD11b^{hi}F4/80^{int}$ cells suppress T cell responses in a NO-dependent manner [4]. After sorting, 80–95% of cells were $Gr1^{hi}CD11b^{hi}$, expressing $F4/80^{int}$ and high levels of monocyte marker Ly6C, with a contamination of 2–13% of $Ly6C^{int}F4/80^{neg}$ cells (Fig. 1C), identified as PMNs by cytospin analysis (see Fig. 3A) and 1–7% of $F4/80^{hi}$ macrophages.

MDSCs produce NO and suppress T cell proliferation

To investigate whether cells elicited by *L. major* infection have the properties of MDSCs, sorted $Gr1^{hi}CD11b^{hi}F4/80^{int}$ cells from injected mice were assayed for NO production and suppression of T cell proliferation (**Fig. 2**). High amounts of NO were detected in cocultures of sorted cells and anti-CD3 or antigen-stimulated LN cells from infected mice (Fig. 2, left panels). Moreover, sorted MDSCs suppressed polyclonal and antigen-specific proliferation in stimulated LNs from infected mice in a NO-dependent manner (Fig. 2, middle panels). Production of type 1 cytokine IFN-γ was not as affected as proliferation to polyclonal or antigen stimulation (Fig. 2, right panels). Therefore, *L. major* infection elicits NO-producing MDSCs that suppress T cell proliferation.

MDSCs as host and effector cells in *L. major* infection

Next, we evaluated the role of MDSCs as host and effector cells in *L. major* infection. Sorted $Gr1^{hi}CD11b^{hi}F4/80^{int}$ cells induced by *L. major* infection had the appearance of immature monocytes and myeloid precursors with ring-shaped nuclei with the presence of 8% contaminant granulocytes (**Fig. 3A**). As only 1–2% of immature monocytes recruited by i.p. injection of *L. major* remained infected with parasites upon 24 h (Fig. 3A, insets, and ref. [17]), sorted monocytes were infected further with *L. major* during 3 h for in vitro killing assays. About 40% of monocytes became infected upon in vitro incubation with *L. major* promastigotes (Fig. 3B). After 48 h, however, only 20% of monocytes remained infected (Fig. 3B). To assess the mechanism of parasite killing, we used the iNOS inhibitor L-NIL [33, 34], which increased the proportion of infected monocytes (Fig. 3B). By contrast, treatment with IFN-γ and IL-4 increased NO production (not shown) and reduced the number of amastigotes within each cell (Fig. 3C). As summarized in Fig. 3D, cultured monocytes killed ~50% of parasites compared with recently infected cells, whereas monocytes incubated with L-NIL were not able to control parasite infection, even upon treatment with IFN-γ and IL-4. Therefore, acutely recruited MDSCs are able to kill *L. major* parasites in a NO-dependent manner. We also injected *L. major* in mouse footpads in the presence or absence of sorted $Gr1^{hi}CD11b^{hi}F4/80^{int}$ monocytes. Parasite burden was assessed 2 weeks later, when differences in lesion measures in footpads,

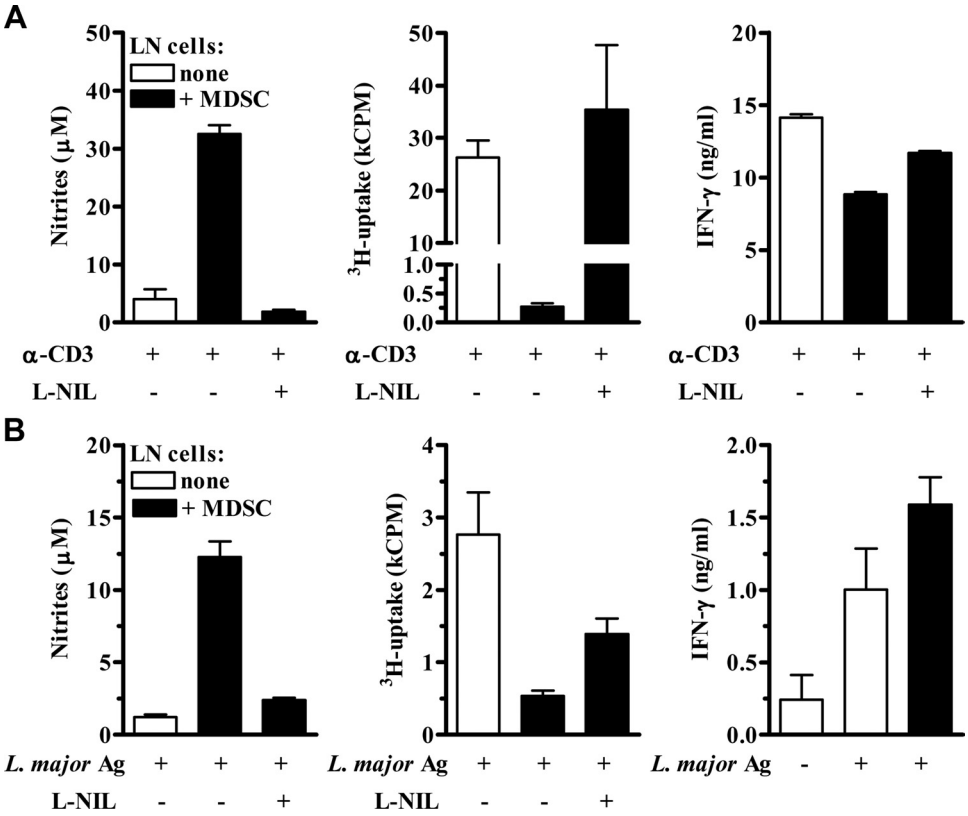

Figure 2. Suppression of T cell proliferation by MDSCs. (A and B) LN cells from 2 week-infected mice were cultured in triplicates with medium only or stimulated with (A) anti-CD3 or (B) *L. major* antigens in the presence (1:1) or absence of $Gr1^{hi}CD11b^{hi}F4/80^{int}$ sorted monocytes from mice injected with *L. major*. L-NIL was added where indicated to inhibit NO production. (Left) NO was detected in 48-h culture supernatants. (Middle) Proliferation was assessed by incorporation of thymidine. kCPM, Thousands of cpm. (Right) IFN-γ was assessed by ELISA. Results represent two independent experiments.

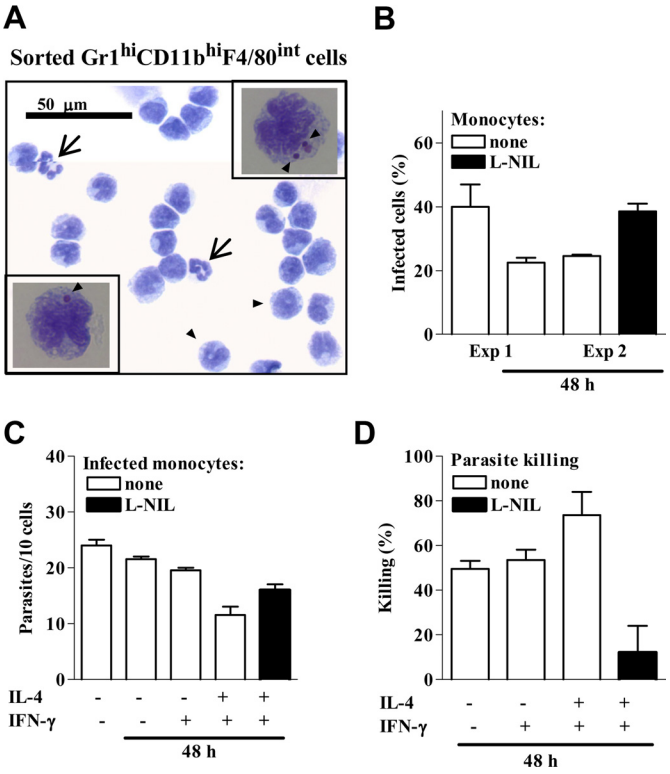

Figure 3. MDSCs express NO-dependent parasite killing. (A) Sorted $Gr1^{hi}CD11b^{hi}F4/80^{int}$ cells (92% pure) show morphology of monocytes and myeloid precursors with ring-shaped nuclei (arrowheads) and among mononuclear cells, a few contaminating (arrows) PMNs (original magnification, 400×). Insets display cells infected with *L. major* amastigotes, indicated by arrowheads (original magnification, 1000×). (B–D) Sorted cells were infected with *L. major* promastigotes and cultured in duplicates with IFN-γ, IL-4, and L-NIL as indicated. Slides were processed immediately upon infection (Day 0) or upon 48 h (Day 2). (B) The proportion of monocytes infected with *L. major* amastigotes is represented. (C) Graph displays the number of amastigotes/10 infected monocytes. (D) Parasite killing was based on reduction in the number of parasites relative to 100 monocytes between Days 0 and 2 for each treatment. Results are representative of two independent experiments.

infected in the presence or absence of monocytes, became apparent (**Fig. 4A**). Injection of monocytes reduced lesions (Fig. 4A) and parasite loads in draining LNs (Fig. 4B), suggesting that monocytes help to control infection in vivo. Secondary to lower dissemination of parasites to LNs, proliferation to *L. major* antigens was also reduced (Fig. 4D), despite an intact polyclonal response (Fig. 4C). Therefore, injection of $Gr1^{hi}CD11b^{hi}F4/80^{int}$ monocytes prevented development of lesions and reduced parasite burden, although contaminant granulocytes and macrophages may also contribute to the observed effects [35–37].

Maturation of MDSCs promotes *L. major* infection

We also investigated how differentiation of immature monocytes into macrophages affects immunity to *L. major*. For that, total peritoneal cells elicited by *L. major* infection were treated with 2 μM ATRA, which induces MDSCs to differentiate into mature macrophages [29] (**Fig. 5A**). Treatment with ATRA also reduced cytokine-induced NO production (Fig. 5B) and increased parasite replication within macrophages, even in the presence of IFN-γ (Fig. 5C). Similar results were observed in sorted $Gr1^{hi}CD11b^{hi}F4/80^{int}$ cells treated with ATRA (Fig. 5D, and data not shown). Next, mice were infected with *L. major* in both footpads, which were treated with ATRA or control vehicle (**Fig. 6**). Remarkably, injection of *L. major* and ATRA increased footpad lesions (Fig. 6A) and parasite burden in LNs (Fig. 6B). Furthermore, treatment with ATRA reduced spontaneous NO production (Fig. 6C) and reversed suppression of T cell proliferation in draining LNs (Fig. 6D). Although we cannot discard in vivo effects of ATRA on other cells, such as Tregs [38], in vitro assays with MDSCs alone reproduce features of injection of ATRA in infected mice (Figs. 5D and 6).

Here, we show that peritoneal injection of *L. major* elicited immature monocytes expressing $CD11b^{hi}$, $Gr1^{hi}(Ly6C^{hi})$, $F4/80^{int}$ and NO, which were able to kill intracellular parasites by a NO-dependent mechanism and reduce parasite burden in vivo. On the other hand, $Gr1^{+}$ monocytes, recently recognized as effector cells in *L. major* infection, quickly destroy parasites

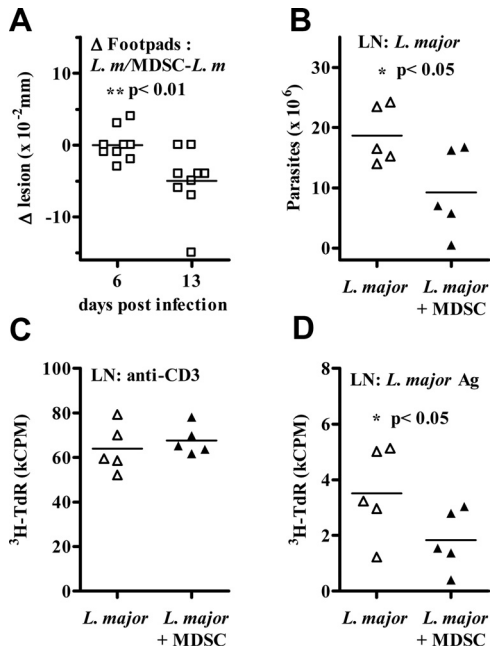

Figure 4. MDSCs help to control *L. major* infection. Mice were injected in the right footpad with *L. major* (*L. m*) and in the left footpad with *L. major* and sorted $Gr1^{hi}CD11b^{hi}F480^{int}$ monocytes. (A) Differences (Δ=left–right) between footpad measures were depicted for each mouse, 6 and 13 dpi. A significant difference between repeated measures, analyzed in two independent experiments, was indicated (**$P<0.01$; $n=9$ mice/group). (B) Parasite burden was evaluated in LNs for each mouse. (C and D) Proliferation of LN cells to (C) anti-CD3 and (D) *L. major* antigens. Significant differences (*$P<0.05$; $n=5$ mice/group) occurred between LN draining footpads infected only (△) and infected footpads treated with MDSCs (▲). Results are representative of two independent experiments.

in a phox-dependent manner, although other leishmanicidal mechanisms were not discarded [17].

In our model, the same NO-dependent mechanism that kills parasites is involved in suppression of T cell proliferation by MDSCs. Cross-talking between T cells and MDSCs includes activated T cells producing IFN-γ to induce NO production by MDSCs, which in turn, suppress T cell proliferation. Similarly, $Gr1^{hi}CD11b^{+}$ myeloid suppressor cells cause defective T cell proliferation in spleens of mice infected with *T. cruzi* in an IFN-γ and NO-dependent manner [11]. IFN-γ produced by T cells is involved in induction of NO production by MDSCs [4, 5, 11] and perhaps in mobilization of immature myeloid precursors in *T. cruzi* infection [11]. In addition, type 2 cytokines, IL-4 and IL-13, share IL-4Rα signaling and may synergize with IFN-γ to activate NO production by MDSCs [5].

Altogether, these results indicate that $Gr1^{hi}CD11b^{hi}F4/80^{int}$ MDSCs contribute as effector cells for immunity to *L. major* infection, despite their suppressor activity. Interventions, such as modulation of host cytokines, which promote generation and activation of MDSCs, might improve clearance of parasites and resolution of lesions. It should be noted, however, that monocytes mature into small macrophages [39] or DCs [40] and as immature macrophages, express plasticity to differentiate into classically or alternatively activated macrophages, depending on relative activities of cytokines and the presence of apoptotic cells and pathogens [5, 41].

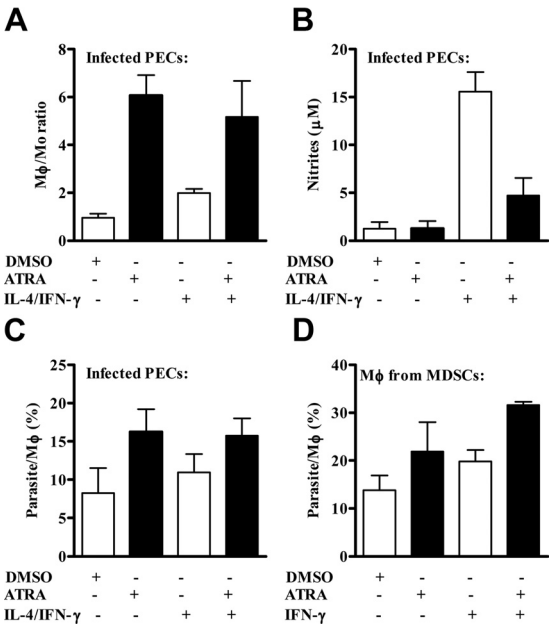

Figure 5. ATRA induces maturation of MDSCs. (A–C) PECs or (D) sorted monocytes were infected and cultured in triplicates in the presence or absence of ATRA, IFN-γ, and IL-4, as indicated, during 48 h. Slides were stained and evaluated for (A) cell morphology and (C and D) macrophage infection. Mϕ, macrophages; Mo, Monocytes. (B) NO was detected in 48-h culture supernatants. Results are representative of two (D) or three independent experiments.

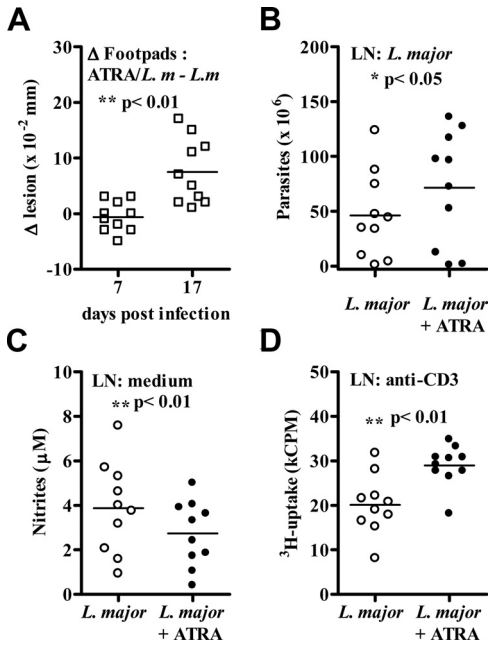

Figure 6. Treatment with ATRA promotes *L. major* infection. Mice were injected in the right footpad with *L. major* and DMSO and in the left footpad with *L. major* and ATRA. (A) Differences (Δ) between measures in left and right footpads were depicted for each mouse, 7 and 17 dpi. (B) Parasite burden was evaluated in LN draining lesions for each mouse. (C) Spontaneous NO production in LN cultures. (D) Proliferation of LN cells to anti-CD3. Results were analyzed by paired *t* test, and significant differences were indicated (**P*<0.05; ***P*<0.01). Results were collected in two independent experiments (*n*=10 mice/group).

AUTHORSHIP

W.F.P. performed and designed experiments, analyzed data, and cowrote the manuscript; F.L.R-G., L.V.C.G., and N.S.V. performed experiments; F.M. helped in cell sorting; G.A.D. contributed to interpretation of data and organization of the manuscript; and M.F.L. designed the research, supervised the experiments, analyzed data, and wrote the manuscript.

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REFERENCES

1. Gabrilovich, D. I., Nagaraj, S. (2009) Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* **9,** 162–174.
2. Ostrand-Rosenberg, S., Sinha, P. (2009) Myeloid-derived suppressor cells: linking inflammation and cancer. *J. Immunol.* **182,** 4499–4506.
3. Ribechini, E., Greifenberg, V., Sandwick, S., Lutz, M. B. (2010) Subsets, expansion and activation of myeloid-derived suppressor cells. *Med. Microbiol. Immunol. (Berl.)* **199,** 273–281.
4. Zhu, B., Bando, Y., Xiao, S., Yang, K., Anderson, A. C., Kuchroo, V. K., Khoury, S. J. (2007) CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. *J. Immunol.* **179,** 5228–5237.
5. Gallina, G., Dolcetti, L., Serafini, P., De Santo, C., Marigo, I., Colombo, M. P., Basso, G., Brombacher, F., Borrello, I., Zanovello, P., Bicciato, S., Bronte, V. (2006) Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J. Clin. Invest.* **116,** 2777–2790.
6. Geissmann, F., Jung, S., Littman, D. R. (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* **19,** 71–82.
7. Sunderkötter, C., Nikolic, T., Dillon, M. J., Van Rooijen, N., Stehling, M., Drevets, D. A., Leenen, P. J. (2004) Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J. Immunol.* **172,** 4410–4417.
8. Dunay, I. R., Damatta, R. A., Fux, B., Presti, R., Greco, S., Colonna, M., Sibley, L. D. (2008) Gr1(+) inflammatory monocytes are required for mucosal resistance to the pathogen *Toxoplasma gondii*. *Immunity* **29,** 306–317.
9. Robben, P. M., LaRegina, M., Kuziel, W. A., Sibley, L. D. (2005) Recruitment of Gr-1+ monocytes is essential for control of acute toxoplasmosis. *J. Exp. Med.* **201,** 1761–1769.
10. Voisin, M. B., Buzoni-Gatel, D., Bout, D., Velge-Roussel, F. (2004) Both expansion of regulatory GR1+ CD11b+ myeloid cells and anergy of T lymphocytes participate in hyporesponsiveness of the lung-associated immune system during acute toxoplasmosis. *Infect. Immun.* **72,** 5487–5492.
11. Goñi, O., Alcaide, P., Fresno, M. (2002) Immunosuppression during acute *Trypanosoma cruzi* infection: involvement of Ly6G (Gr1(+))CD11b(+) immature myeloid suppressor cells. *Int. Immunol.* **14,** 1125–1134.
12. Cuervo, H., Guerrero, N. A., Carbajosa, S., Beschin, A., De Baetselier, P., Girones, N., Fresno, M. (2011) Myeloid-derived suppressor cells infiltrate the heart in acute *Trypanosoma cruzi* infection. *J. Immunol.* **187,** 2656–2665.
13. dos Santos, M. S., Vaz Cardoso, L. P., Nascimento, G. R., Lino Rde Jr., S., Dorta, M. L., de Oliveira, M. A., Ribeiro-Dias, F. (2008) *Leishmania major*: recruitment of Gr-1+ cells into draining lymph nodes during infection is important for early IL-12 and IFN γ production. *Exp. Parasitol.* **119,** 403–410.
14. Beil, W. J., Meinardus-Hager, G., Neugebauer, D. C., Sorg, C. (1992) Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice. *J. Leukoc. Biol.* **52,** 135–142.
15. Sunderkötter, C., Kunz, M., Steinbrink, K., Meinardus-Hager, G., Goebeler, M., Bildau, H., Sorg, C. (1993) Resistance of mice to experimental leishmaniasis is associated with more rapid appearance of mature macrophages in vitro and in vivo. *J. Immunol.* **151,** 4891–4901.
16. Steinbrink, K., Schonlau, F., Rescher, U., Henseleit, U., Vogel, T., Sorg, C., Sunderkotter, C. (2000) Ineffective elimination of *Leishmania major* by inflammatory (MRP14-positive) subtype of monocytic cells. *Immunobiology* **202,** 442–459.
17. Goncalves, R., Zhang, X., Cohen, H., Debrabant, A., Mosser, D. M. (2011) Platelet activation attracts a subpopulation of effector monocytes to sites of *Leishmania major* infection. *J. Exp. Med.* **208,** 1253–1265.
18. Sato, N., Ahuja, S. K., Quinones, M., Kostecki, V., Reddick, R. L., Melby, P. C., Kuziel, W. A., Ahuja, S. S. (2000) CC chemokine receptor (CCR)2 is required for Langerhans cell migration and localization of T helper cell type 1 (Th1)-inducing dendritic cells. Absence of CCR2 shifts the *Leishmania major*-resistant phenotype to a susceptible state dominated by Th2 cytokines, B cell outgrowth, and sustained neutrophilic inflammation. *J. Exp. Med.* **192,** 205–218.
19. Quinones, M. P., Estrada, C. A., Jimenez, F., Martinez, H., Willmon, O., Kuziel, W. A., Ahuja, S. K., Ahuja, S. S. (2007) CCL2-independent role of CCR2 in immune responses against *Leishmania major*. *Parasite Immunol.* **29,** 211–217.
20. Conrad, S. M., Strauss-Ayali, D., Field, A. E., Mack, M., Mosser, D. M. (2007) *Leishmania*-derived murine monocyte chemoattractant protein 1 enhances the recruitment of a restrictive population of CC chemokine receptor 2-positive macrophages. *Infect. Immun.* **75,** 653–665.
21. Mirkovich, A. M., Galelli, A., Allison, A. C., Modabber, F. Z. (1986) Increased myelopoiesis during *Leishmania major* infection in mice: generation of "safe targets", a possible way to evade the effector immune mechanism. *Clin. Exp. Immunol.* **64,** 1–7.
22. Goto, Y., Sanjoba, C., Arakaki, N., Okamoto, M., Saeki, K., Onodera, T., Ito, M., Matsumoto, Y. (2007) Accumulation of macrophages expressing MRP8 and MRP14 in skin lesions during *Leishmania major* infection in BALB/c and RAG-2 knockout mice. *Parasitol. Int.* **56,** 231–234.
23. Sacks, D., Noben-Trauth, N. (2002) The immunology of susceptibility and resistance to *Leishmania major* in mice. *Nat. Rev. Immunol.* **2,** 845–858.
24. McMahon-Pratt, D., Alexander, J. (2004) Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? *Immunol. Rev.* **201,** 206–224.
25. Alexander, J., Bryson, K. (2005) T helper (h)1/Th2 and *Leishmania*: paradox rather than paradigm. *Immunol. Lett.* **99,** 17–23.
26. Lykens, J. E., Terrell, C. E., Zoller, E. E., Divanovic, S., Trompette, A., Karp, C. L., Aliberti, J., Flick, M. J., Jordan, M. B. (2010) Mice with a selective impairment of IFN-γ signaling in macrophage lineage cells demonstrate the critical role of IFN-γ-activated macrophages for the control of protozoan parasitic infections in vivo. *J. Immunol.* **184,** 877–885.
27. Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M., Sacks, D. L. (2002) CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* **420,** 502–507.
28. Sacks, D., Anderson, C. (2004) Re-examination of the immunosuppressive mechanisms mediating non-cure of *Leishmania* infection in mice. *Immunol. Rev.* **201,** 225–238.
29. Nefedova, Y., Fishman, M., Sherman, S., Wang, X., Beg, A. A., Gabrilovich, D. I. (2007) Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res.* **67,** 11021–11028.
30. Titus, R. G., Marchand, M., Boon, T., Louis, J. A. (1985) A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. *Parasite Immunol.* **7,** 545–555.
31. Ribeiro-Gomes, F. L., Moniz-de-Souza, M. C., Borges, V. M., Nunes, M. P., Mantuano-Barradas, M., D'Avila, H., Bozza, P. T., Calich, V. L., DosReis, G. A. (2005) Turnover of neutrophils mediated by Fas ligand drives *Leishmania major* infection. *J. Infect. Dis.* **192,** 1127–1134.
32. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., Tannenbaum, S. R. (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.* **126,** 131–138.
33. Moore, W. M., Webber, R. K., Jerome, G. M., Tjoeng, F. S., Misko, T. P., Currie, M. G. (1994) L-N6-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. *J. Med. Chem.* **37,** 3886–3888.
34. Stenger, S., Thuring, H., Rollinghoff, M., Manning, P., Bogdan, C. (1995) L-N6-(1-iminoethyl)-lysine potently inhibits inducible nitric oxide synthase and is superior to NG-monomethyl-arginine in vitro and in vivo. *Eur. J. Pharmacol.* **294,** 703–712.
35. Ribeiro-Gomes, F. L., Otero, A. C., Gomes, N. A., Moniz-De-Souza, M. C., Cysne-Finkelstein, L., Arnholdt, A. C., Calich, V. L., Coutinho, S. G., Lopes, M. F., DosReis, G. A. (2004) Macrophage interactions with neutrophils regulate *Leishmania major* infection. *J. Immunol.* **172,** 4454–4462.
36. Peters, N. C., Egen, J. G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., Lawyer, P., Fay, M. P., Germain, R. N., Sacks, D. (2008) In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* **321,** 970–974.
37. Peters, N. C., Sacks, D. L. (2009) The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis. *Cell. Microbiol.* **11,** 1290–1296.
38. Benson, M. J., Pino-Lagos, K., Rosemblatt, M., Noelle, R. J. (2007) All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J. Exp. Med.* **204,** 1765–1774.
39. Ghosn, E. E., Cassado, A. A., Govoni, G. R., Fukuhara, T., Yang, Y., Monack, D. M., Bortoluci, K. R., Almeida, S. R., Herzenberg, L. A. (2010) Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc. Natl. Acad. Sci. USA* **107,** 2568–2573.
40. Serbina, N. V., Salazar-Mather, T. P., Biron, C. A., Kuziel, W. A., Pamer, E. G. (2003) TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* **19,** 59–70.
41. Filardy, A. A., Pires, D. R., Nunes, M. P., Takiya, C. M., Freire-de-Lima, C. G., Ribeiro-Gomes, F. L., DosReis, G. A. (2010) Proinflammatory clearance of apoptotic neutrophils induces an IL-12^{low}IL-10^{high} regulatory phenotype in macrophages. *J. Immunol.* **185,** 2044–2050.

KEY WORDS:
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