Mycobacterium bovis Bacillus Calmette-Guérin Vaccination Mobilizes Innate Myeloid-Derived Suppressor Cells Restraining In Vivo T Cell Priming via IL-1R–Dependent Nitric Oxide Production

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Nitric Oxide Production
Restraining In Vivo T Cell Priming via IL-1R–Dependent Mobilizes Innate Myeloid-Derived Suppressor Cells
nonpathogenic myeloid-derived suppressor cells, akin to mouse tumor-infiltrating cells. These propathogenic cells dampen the early T cell response by all-trans retinoid acid treatment increased the number of IFN-γ-producing CD11b+Ly-6CintLy-6G–producing CD4 T cells. Thus, BCG vaccination recruits innate myeloid-derived suppressor cells, akin to mouse tumor-infiltrating cells. These propathogenic cells dampen the early T cell response and might facilitate BCG persistence. The Journal of Immunology, 2010, 184: 2038–2047.

Promptly after pathogen entry or danger signal encounter, early tissue inflammatory infiltrate includes neutrophils, the master cell type involved in controlling invaders, and monocytes (MOs), which mature in dendritic cells (DCs) or tissue macrophages. MOs circulating in the blood are heterogeneous in phenotype and function (1). In the mouse, the different levels of CX3CR1 expression, the fractalkine receptor, and Ly-6C, a glycosphatidylinositol-anchored protein, at the cell surface define two different subsets (2): CX3CR1hiLy-6C– MOs migrate to inflamed tissues upon CCR2 activation of the host response against infection (3), and CX3CR1loLy-6CCCR2– MOs replenish the resident tissue macrophage pool under steady-state conditions (2). Blood MOs with intermediate levels of Ly-6C may represent different differentiation phases of a continuum (4–6) or bona fide MO subsets (7). Bone marrow-derived precursors for neutrophils and MOs (8), sometimes referred to as band-cells or ring-shaped nucleus cells, also circulate under steady-state conditions (9).

Inflammation, intertwined with the innate immune response, becomes involved in adaptive immunity when DCs prime T cells for control of primary infection or for protection against a second pathogen encounter. In response to moderate inflammatory signals, CX3CR1loLy-6CbMOs migrate to the draining lymph node, where they differentiate in DCs and prime the T cell response (10). Upon vaccination, the consequences of these early immune events on protective immunity remain ill defined. Notably, understanding inflammatory/innate response to the tuberculosis vaccine Mycobacterium bovis bacillus Calmette-Guérin (BCG) might give some clues as to why BCG does not consistently protect adults against pulmonary disease (11). We showed that during BCG vaccination in mouse models, neutrophils shuttle BCG from the skin to the draining lymph node (12) and establish cross-talk with DCs to facilitate the presentation of Ag to T cells (13).

Inflammation is a double-edged sword that must be tightly controlled. The first checkpoint occurs during acute inflammation and entails the active coordination of suppression of neutrophil influx and local release of molecular host response machinery. The second checkpoint involves backup processes that dampen the inflammatory T cell responses to avoid chronic inflammation and tissue injury. These include the production of anti-inflammatory cytokines by innate cells and generation of regulatory T cells. Myeloid-derived suppressor cells (MDSCs) suppress T cells in pathologic situations such as cancer (14) or sepsis (15). MDSCs constitute a heterogeneous population of CD11b+Gr1 (Ly-6C/Ly-6G) cells, comprising MOs, granulocytes, and DCs at different stages of differentiation (16). In tumor-bearing mice, MDSCs expand in the blood, spleen, and tumors, where they contribute to tumor-associated Ag-specific T cell suppression and tolerance via NO production (17, 18).

We characterized innate/inflammatory cells infiltrating the skin early after BCG injection and their effects on T cell priming in the draining lymph node in a mouse model of intradermal BCG vaccination. BCG bacillus Calmette-Guérin (BCG) intradermal vaccine remains ill defined. Three days after BCG inoculation into the mouse ear, in addition to neutrophils infiltrating skin, we observed CD11b+Ly-6CintLy-6G–myeloid cells. Neutrophil depletion markedly enhanced their recruitment. These cells differed from inflammatory monocytes and required MyD88-dependent BCG-specific signals to invade skin, whereas neutrophil influx was MyD88 independent. Upon BCG phagocytosis, CD11b+Ly-6CintLy-6G–cells produced NO, which required the IL-1 receptor. Despite NO production, they were unable to kill BCG or the nonpathogenic Mycobacterium smegmatis. However, they markedly impaired T cell priming in the draining lymph node. Their elimination by all-trans retinoid acid treatment increased the number of IFN-γ-producing CD4 T cells. Thus, BCG vaccination recruits innate myeloid-derived suppressor cells, akin to mouse tumor-infiltrating cells. These propathogenic cells dampen the early T cell response and might facilitate BCG persistence. The Journal of Immunology, 2010, 184: 2038–2047.

"Mycobacterium bovis" Bacillus Calmette-Guérin Vaccination Mobilizes Innate Myeloid-Derived Suppressor Cells Restraining In Vivo T Cell Priming via IL-1R–Dependent Nitric Oxide Production

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vaccination. During the first 3 d, after BCG injection, we observed the recruitment of a subset of CD11b⁺ myeloid-derived cells in addition to neutrophils in the skin. These CD11b⁺ cells produced inducible NO synthase (iNOS) but were unable to kill mycobacteria. However, they blocked T cell proliferation and dampened Ag-specific priming in the draining lymph node through NO production in vivo. Our findings thus showed that BCG vaccination recruits innate MDSCs, similar to those infiltrating tumors in mice, that impair the in vivo T cell response.

**Materials and Methods**

**Injection into ear skin in mice and mycobacterial strains**

Female C57BL/6 or BALB/c (Centre d’Elevage Janvier, Le Genest-Saint-Isle, France), IL-1R⁻/⁻ and F1 C57BL/6 × CX3CR1⁻/⁻F4/80⁻/⁻ (Centre de Cyco-ordination, Distribution, Typage et Archivage Animal, Orléans, France) and in house-bred MyD88⁻/⁻, TLR2 × 4⁻⁻, TLR9⁻⁻, and OT-II mice were housed in our animal care facility under specific pathogen-free conditions. After anesthesia with 5 µl xylazine 2% (v/v) and 1 µg ketamine, each mouse received 10 µl culture containing 10⁶ CFUs of mycobacteria per ear dorsum. Strains used were BCG Pasteur 1173P2, 100 mM HEPES, and 100 µM L-NG-monomethyl arginine citrate (L-NMMA; Sigma-Aldrich, St. Louis, MO) was added to stimulated cells for 36 h (Sigma-Aldrich) was added to stimulated cells for 36 h and IFN-γ spot forming cells (SPCs) were analyzed with the IFN-γ ELISPOT assay (BD Biosciences, San Jose, CA). For allogeneic MLR, BALB/c spleen T cells were positively selected using anti-CD3 (17A2, BD Pharmingen, San Diego, CA) and magnetic bead selection (Miltenyi Biotec). BALB/c T cells (10⁵) were mixed with 2.5 × 10⁵ to 10⁵ C57BL/6 SkEx cells. Alternatively, a fixed number of 4 × 10⁵ C57BL/6 SkEx cells was added to 2.5 × 10⁵ to 10⁵ C57BL/6 bone marrow-derived BCG-activated DCs fixed with 2% (v/v) paraformaldehyde and 10⁻⁵ BALB/c T cells. After 72 h culture, 0.5 µCi [³H]thymidine was added in each well for the last 8 h. Under similar conditions, IFN-γ production in the 36-h cell supernatant was measured by ELISA (R&D Systems, Minneapolis, MN).

**Flow cytometry**

Single suspensions were stained for surface markers for 30 min at 4°C after blocking Fc receptors with anti-CD16/CD32 (2.4G2), in PBS containing 0.1% (v/v) sodium azide and 10% (v/v) FCS. Cells were stained with Abs against CD11b (M1/70,106, Ly-6C (AL-21), Ly-6G (1A8), CD11c (HL3), L4, IIB4(29) and CD31 (MEC 13.3) from BD Pharmingen or against F4/80 (A3-1, Serotec, Oxford, U.K.), CD115 (AFS98, eBioscience, San Diego, CA). For intracellular staining, cells were permeabilized with Cytofix-Cytoperm kit (554714, BD Pharmingen) and incubated with rabbit polyclonal anti-NO synthase 2 (NOS2) (SA-200, Biomol International, Plymouth Meeting, PA) followed with Alexa Fluor 594-conjugated goat anti-rabbit IgG (Molecular Probes) or with anti-TNF (BD Biosciences). Data were analyzed using FACS caliber; 3 × 10⁶ events were collected and analyzed with FlowJo software (BD Bioscience).

**Immunohistochemistry**

In situ expression of Ly-6C⁺ was assessed in ear skin snap-frozen and embedded in 1.5 mm optimum cutting temperature compound (Tissue Tek, Sakura, Torrance, CA). Cryostat sections (5–8 µm) were fixed in cold 80% (v/v) acetone and 20% (v/v) ethanol, air dried, and blocked for 30 min in BSA containing medium (Vector Laboratories, Burlingame, CA). Sections were incubated with anti-Ly-6C and Alexa Fluor 633- or 594-conjugated goat anti-rabbit IgG (Molecular Probes). Rabbit polyclonal anti-NOS2 and Alexa Fluor 594- or 488-conjugated goat anti-rabbit IgG were used for NOS2 detection.

**Microscope and image acquisition**

For immunohistochemistry on tissues, images were acquired on microscope (Axiostar, Zeiss, Oberkochen, Germany) with objective (Achromat 20×/0.45, Zeiss) using a camera (Leica Camera AG, Solms, Germany) and Leica Qwin software (Leica) or on a confocal microscope equipped with a blue laser diode (405 nm), an Argon laser (488 nm), helium-neon laser (543 nm) and objective (Plan Apochromat 63×/1.4 NA, Zeiss) with LSM 510 V3.2 software (Zeiss). For cytocentrifuged cells, images were acquired on a light microscope (Axiostar, Zeiss) equipped with an oil iris objective (Achromat 100×/1.25 NA, Zeiss), with a camera (Leica 300F) and Qwin software (Leica). For fluorescence microscopy on SkEx cells, images were acquired on a microscope (eclipse E600; Nikon, Melville, NY) equipped with an objective (Plan Apochromat 60×/1.40, Nikon) with camera (DXM 1200F, Nikon) using imaging software (Nikon, NIS document BR). All images were transferred to Adobe Photoshop (Adobe Systems, San Jose, CA) for editing figures.

**Statistical analysis**

Values are expressed as mean ± SD unless otherwise indicated. The Kruskal-Wallis test was used first to ascertain that significant variance existed among the groups studied. The Mann-Whitney U test was then used to test statistical significance of the differences between two groups, and p values (indicated on each figure) of less than 0.05 were considered significant. All calculations were made using Prism 5.0 software (GraphPad, San Diego, CA).

**Results**

CD11b⁺ Ly-6C⁺Ly-6G⁻ SkEx cells accumulate early in skin and blood after intradermal BCG

C57BL/6 mice were inoculated in each ear dorsum with 10⁶ BCG CFUs. After 24 and 72 h, cells migrating from ear SkExs (23) were analyzed ex vivo. SkEx cells were almost exclusively CD11b⁺Ly-6C⁺Ly-6G⁻ neutrophils (2, 5) from CD11b⁺Ly-6C⁺Ly-6G⁻
cells. Neutrophils were the predominant SkEx cell type after 24 h (Fig. 1A), as described previously (12). However, a significant proportion of CD11b$^+$Ly-6G$^-$ cells uniformly expressed intermediate levels of Ly-6C at the surface (Ly-6C$^{int}$) and had a heterogeneous nuclear morphology (data not shown). Whereas the absolute numbers of neutrophils remained constant, CD11b$^+$ Ly-6C$^{int}$Ly-6G$^-$ cell numbers increased (Fig. 1B). Few CD11b$^+$ cells were recruited after injection of PBS into the skin (data not shown). CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ cells expressed surface MHC class II and F4/80 and low levels of the myeloid marker CD31. They were negative for CD115 and CD11c (Fig. 1C) and thus were not DCs or MOs. Given that a major inflammatory MO subset has been defined on the basis of CX3CR1 and Ly-6C levels at the cell surface (7), we used CX3CR1$^{GFP}$ knockin mice (24) to decipher inflammatory MO recruitment in skin following BCG injection. No CX3CR1 expressing cells were observed among CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ SkEx cells 24 h after BCG injection (Fig. 1D). The number of CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ SkEx cells observed in the ear dermis after BCG inoculation did not differ between CX3CR1$^{GFP/GFP}$ knockout and CX3CR1$^{+/GFP}$ knockin mice (Fig. 1E). This suggested that CX3CR1$^+$ did not control their recruitment. Thus, myeloid cells, distinct from neutrophils, DCs, and MOs, the major actors during the inflammatory response, were recruited early after BCG vaccination in skin.

In order to determine the origin of CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ skin cells, CX3CR1$^{GFP/GFP}$ mouse peripheral blood CD11b$^+$ cells were analyzed 24 h and 72 h after BCG vaccination. After gating out neutrophils, we found blood CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$CX3CR1$^+$ cells in addition to the two expected CX3CR1$^+$ MO subsets. These cells were detected also in mock-injected animals albeit to smaller numbers.

**FIGURE 1.** BCG injection induces early accumulation of CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ cells, which do not belong to CX3CR1 monocyte subsets, in skin and blood. A, Cells migrating from SkEx 24 h and 72 h after BCG injection were analyzed by flow cytometry. CD11b$^+$ cells contained Ly-6C$^+$Ly-6G$^+$ neutrophils and Ly-6C$^{int}$Ly-6G$^-$ subsets. B, CD11b$^+$Ly-6G$^-$, Ly-6C$^+$ neutrophils, and CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ cells from 24 and 72 h SkEx cells were counted. The number of neutrophils remained constant, whereas CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ cells increased in number (mean ± SD cell number per mouse; n = 5 experiments; p = 0.0079, Mann-Whitney U test). C, Surface expression of CD11c, MHC class II, F4/80, and CD115 (filled histograms) on CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ cells 72 h after BCG injection in the ear. Open histograms represent isotype controls. D, Characterization of CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ cells pooled from five CX3CR1$^{+/GFP}$ mice 72 h after BCG injection in the ear (gate R1). CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ cells were negative for CX3CR1 (GFP), and the number of migrating CD11b$^+$Ly-6C$^{int}$Ly-6G$^-$ cells was identical in CX3CR1$^{+/GFP}$ knockin and CX3CR1$^{GFP/GFP}$ knockout mice (E). F, Two CX3CR1$^+$ MO populations were distinguished from peripheral blood CD11b$^+$Ly-6G$^-$ gated cells. The Ly-6C$^{int}$ population that increased in number upon BCG injection did not express CX3CR1. Cytocentrifuged cells were observed under a light microscope harbored ring-shaped nuclei (R1, May-Grunwald-Giemsa stain). Bar, 10 μM; original magnification ×100.
levels. They displayed ring-shaped nuclei, as previously described for immature myeloid cells (9) (Fig. 1F). Thus, CD11b+Ly-6CintLy-6G cells, that increased in blood and migrated to skin early in response to BCG, were distinct from the CX3CR1hiLy-6Cint and CX3CR1hiLy-6Chi MO subsets circulating in mouse blood (2).

**Neutrophil depletion increases influx of CD11b+Ly-6CintLy-6G cells to skin**

Upon acute inflammation, an initial massive influx of neutrophils is followed by the recruitment of MOs, which help to resolve inflammation. The nature of extravasation kinetics (25) and interplay between the different subsets remains unclear (26, 27). We examined the effect of neutrophils on the recruitment of CD11b+Ly-6CintLy-6G cells to the dermis in CX3CR1-GFP mice. Neutrophil-depleting Abs NIMP-R14 (rat IgG2b) (21) and 1A8 (20) were injected i.p. during the first 72 h after BCG injection. Both treatments induced neutrophil depletion among SkEx cells and stimulated the recruitment of CD11b+Ly-6CintLy-6G cells (Supplemental Fig. 1). In SkEx cells from neutrophil-depleted CX3CR1+/GFP mice, we only observed CD11b+Ly-6CintLy-6G cells 24 and 72 h after BCG injection (Fig. 2A). Their numbers were 2.1 times and 2.9 times higher at 24 and 72 h after neutrophil depletion than in isotype-injected control mice (Fig. 2B). We did not detect CX3CR1+GFP SkEx cells at these early time points (data not shown), although we observed CX3CR1+ SkEx cells by day 25 following BCG injection in the ear (Supplemental Fig. 2). Neutrophil-depleted C57/BL6 mice were injected with the BCG strain Myc409 producing EGFP (12). We did not detect Ly-6C+ cells in ear cryosections 4 h after BCG injection in these mice. By contrast, large numbers of Ly-6C+ cells infiltrated the dermis between 24 and 72 h (Fig. 2C), indicating that these cells were recruited from the periphery. These cells mostly colocalized with BCG (Fig. 2C). Therefore, CD11b+Ly-6CintLy-6G myeloid cells recruited to skin early after BCG injection were increased by neutrophil depletion.

**BCG-recruited CD11b+Ly-6CintLy-6G− myeloid cells are specialized for iNOS production**

Seventy-two hours after BCG (Myc409) inoculation in C57BL/6 ear skin, both SkEx neutrophils and CD11b+Ly-6CintLy-6G− cells showed similar levels for EGFP staining, suggesting that they have similar capacities to phagocytose BCG (Fig. 3A). Mouse neutrophils produce TNF in response to BCG infection (13). Similar levels of intracellular TNF staining were observed for neutrophils and CD11b+Ly-6CintLy-6G− cells after BCG infection (Fig. 3A). Only CD11b+Ly-6CintLy-6G− cells showed intense staining for iNOS (Fig. 3B, in red), an enzyme playing a major role in mycobacterial control. Whereas few iNOS+ cells were detected in nondepleted mice, almost all SkEx cells displayed high iNOS staining in neutrophil-depleted mice. Accordingly, NO production by SkEx cells was eight times higher in neutrophil-depleted than in control mice (Fig. 3C). In situ analysis revealed colocalization of Ly-6C+ cells (blue) with iNOS (red) and BCG (Myc409, green) (Fig. 3D). Intracellular iNOS was analyzed in blood cells from CX3CR1+GFP mice 72 h after BCG injection. No iNOS+ cells were detected in mouse blood when PBS was injected in ear (data not shown). The two CX3CR1+ blood MO subsets were negative for iNOS. Intracellular iNOS was detected in CD11b+Ly-6CintLy-6G−CX3CR1− cells only in the presence and absence of

**FIGURE 2.** Neutrophil depletion increased the accumulation of CD11b+Ly-6CintLy-6G− cells in skin and blood in response to BCG. A. Five mice were depleted for neutrophils by NIMP-R14 injection before and after BCG inoculation. Control mice were treated with HB152 control isotype. Ly-6C and Ly-6G surface expression on SkEx cells was analyzed 24 and 72 h after BCG injection and counted from five pooled mice (mean ± SD cell number per mouse; n = 3 experiments) (B). C. Ear cryosections from neutrophil-depleted mice CD11b+Ly-6CintLy-6G− cells 4, 24, and 72 h after green fluorescent BCG (Myc409) injection were immunostained with anti–Ly-6C (red), and Ly-6C+ cells were absent in skin 4 h after BCG (Myc409) injection. Large numbers of these cells were then recruited by 72 h and colocalized with BCG (Myc409, yellow). Bars, 50 μM; original magnification ×20.
FIGURE 3. CD11b\(^+\)Ly-6C\(^{int}\)Ly-6G\(^{2}\) cells recruited to skin, in addition to neutrophils, phagocytose BCG, secrete TNF, and express high levels of iNOS. A, BCG (Myc409) producing eGFP was injected into the mouse ear dorsum, and SkEx cells were analyzed 72 h later. Of migrating CD11b\(^+\)Ly-6C\(^{int}\)Ly-6G\(^{2}\) cells and neutrophils, 62% were infected with BCG, suggesting that both subsets have a similar phagocytic capacity. Similar levels of intracellular TNF were detected for the two subsets. B, Intracellular cytosolic iNOS (red) was detected in SkEx cells under a fluorescence microscope with DAPI-stained nuclei (blue) from neutrophil-depleted (NIMP-R14-treated) and mice treated with HB152 control isotype 72 h after BCG injection. C, NO secreted by SkEx cells pooled from five mice injected with control isotype HB152 or neutrophil-depleting NIMP-R14 was detected by Griess reagent (mean ± SD; n = 3 experiments; p = 0.031, Student t test). D, Cells infiltrating the ear tissue were analyzed in cryosections from neutrophil-depleted mice 72 h after BCG (Myc409, green) injection. Ly-6C\(^{+}\) cells (blue) infiltrating the skin show strong staining for iNOS (red). On merged images, iNOS colocalized with Ly-6C and BCG (Myc409). E, Among blood CD11b\(^+\)Ly-6G\(^{2}\) gated cells from CX3CR1\(^+\)GFP mice, intracellular iNOS was only detected in CD11b\(^+\)Ly-6C\(^{int}\)Ly-6G\(^{2}\) cells (R1 gate). The two CX3CR1\(^+\) MO subsets (R2 and R3 gates) were negative for iNOS. CD11b\(^+\)Ly-6C\(^{int}\)Ly-6G\(^{2}\) cells producing iNOS were detected in blood in the presence (HB152 control isotype injection) and absence (NIMP-R14 injection) of neutrophils, although a higher number of these cells were found in the neutrophil-depleted mice.
neutrophils (Fig. 3E) upon BCG injection, illustrating the specificity of these myeloid cells to produce iNOS both in blood and tissues.

Despite high NO production, innate CD11b+Ly-6CintLy-6G− myeloid cells do not kill mycobacteria

NO is a major antimycobacterial molecule in the mouse. We therefore studied BCG killing by CD11b+Ly-6CintLy-6G− cells. Despite their high numbers in skin from neutrophil-depleted animals, the BCG load 4 h after injection did not decrease for the next 3 (Fig. 4A) or 4 d (data not shown). Both BCG and M. smegmatis belong to the mycobacterial genus. However, whereas the slow-growing BCG is resistant to the hostile macrophage NO-rich environment, the fast-growing M. smegmatis is rapidly killed. CD11b+Ly-6CintLy-6G− iNOS+ cells were purified from SKEx cells from neutrophil-depleted BCG injected mice and incubated in vitro with M. smegmatis for 36 h. Similar M. smegmatis CFUs were recovered from infected CD11b+Ly-6CintLy-6G− iNOS+ cells and from the medium (Fig. 4B). Therefore, despite high levels of NO production, CD11b+Ly-6CintLy-6G− cells did not kill pathogenic or nonpathogenic mycobacteria, whereas classically activated macrophages did (Supplemental Fig. 3).

CD11b+Ly-6CintLy-6G− cells respond to BCG and MyD88/IL-1R signals to invade skin and express maximum iNOS

To determine which signals, on the host and microbial side, might regulate the recruitment of CD11b+Ly-6CintLy-6G− cells and iNOS expression, we analyzed the role of MyD88, a major TLR adaptor for mycobacterial control (28, 29). In MyD88−/− mice, CD11b+Ly-6CintLy-6G− cells represented one-third of the total SKEx cells 72 h after BCG injection (Fig. 5A). By contrast, very few CD11b+Ly-6CintLy-6G− cells were present in MyD88−/− mice, in which neutrophil levels were six times higher. In neutrophil-depleted mice, CD11b+Ly-6C−Ly-6G− cells were not detected among MyD88−/− SKEx cells 24 h after BCG inoculation (data not shown) and were detected at levels four times lower than in MyD88+/+ mice 72 h after BCG injection (Fig. 5A). Major innate receptors, upstream from MyD88, instrumental in mycobacterial control include TLR2, TLR9 (30), and IL-1R (31). None of these receptors were involved in the recruitment of CD11b+Ly-6CintLy-6G− cells to the skin in the absence of neutrophils (Fig. 5B). We also found that CCR2, a major receptor for CX3CR1+ MO recruitment, was not involved in this process (data not shown).

The recruitment and full activation of CD11b+Ly-6CintLy-6G− cells may require different signals. We did not find any differences in the levels of iNOS expression in CD11b+Ly-6CintLy-6G− cells between TLR8+/− and TLR2 × 4−/− or TLR9−/− SKEx cells after BCG injection (data not shown). By contrast, despite similar numbers of CD11b+Ly-6CintLy-6G− SKEx recruited cells, iNOS levels were substantially lower in IL-1R−/− mice than in IL-1R+/+ mice (Fig. 5C). Accordingly, NO levels in IL-1R−/− SKEx cells were eight times lower than in IL-1R+/+ SKEx cells (Fig. 5D). Therefore, IL-1R-controlled intrinsic capacity of CD11b+Ly-6CintLy-6G− cells to be activated. On the microbial side, to determine whether CD11b+Ly-6CintLy-6G− cell activation was a general response to mycobacteria, we compared the skin response to BCG and to M. smegmatis. Whereas BCG derives from the slow-growing pathogenic species M. bovis, M. smegmatis is an opportunistic, fast-growing species. Both species share several pathogen-associated molecular patterns, yet only BCG is able to persist in the host. Similar numbers of CD11b+Ly-6CintLy-6G− SKEx cells were observed in CX3CR1+ Gfp mice injected with M. smegmatis or BCG (data not shown). However, intracellular iNOS was barely detected in SKEx cells from M. smegmatis-injected ears (Fig. 5E). Hence, MyD88-mediated signaling appeared to be necessary for CD11b+Ly-6CintLy-6G− cell recruitment. The production of iNOS required a second signal dependent on molecular patterns present in BCG but not in M. smegmatis and involving IL-1R but not TLR2, −4, or −9.

CD11b+Ly-6CintLy-6G− cells suppress T cells in vitro and dampen T cell priming in the lymph node draining BCG vaccination site

To gain further insight into CD11b+Ly-6CintLy-6G− cell function, C57BL/6 SKEx cells were prepared from neutrophil-depleted or control mice 72 h after BCG injection and mixed with BALB/c CD3+ T cells (Fig. 6A). SKEx cells from control mice, including neutrophils, and possibly some professional APCs such as DCs, were able to stimulate allogenic T cells. However, in vitro neutrophil depletion abolished SKEx cell stimulatory activity. Similarly, iNOS-rich SKEx cells from in vivo neutrophil-depleted animals were unable to stimulate T cells. The iNOS inhibitor L-NMMA was added to cell cultures and inhibited SKEx cell-mediated T cell suppression. Fixed numbers of C57BL/6 SKEx cells from BCG-vaccinated mice were then mixed with increasing numbers of bone marrow-derived BCG-activated C57BL/6 DCs to improve stimulatory capacity of BALB/c allogeneic CD3+ T cells (Fig. 6B). SKEx cells were either obtained in vivo from neutrophil-depleted mice or in vitro from neutrophil-depleted cells. In both cases, SKEx cells completely suppressed allogeneic T cell proliferation (Fig. 6B) and IFN-γ secretion (Supplemental Fig. 4) despite the presence of strongly stimulating BCG-activated DCs. This effect was observed even at the highest DC/T cell ratio and was reversed by L-NMMA addition (Supplemental Fig. 4). Hence, BCG vaccination in skin recruited...
CD11b+Ly-6CintLy-6G−iNOS+ innate cells, capable of marked NO-mediated T cell suppression. The phenotype and suppressive function of skin CD11b+Ly-6CintLy-6G− cells were reminiscent of MDSCs previously described under pathological conditions such as in sepsis and cancer (14). We therefore called these cells innate MDSCs (inMDSCs). We also found that jointly recruited neutrophils counterbalanced inMDSC-mediated T cell suppression in vitro.

We then investigated a potential effect of inMDSCs on T cell priming in the draining lymph node. iNOS-producing CD11c− cells were detected in the auricular lymph node by immunohistochemistry 4 and 72 h after BCG injection in the ear skin. iNOS staining under the lymph node capsule was stronger in neutrophil-depleted (NIMP-R14-injected) IL-1R−/− and IL-1R+/− mice 72 h after BCG injection was detected using Griess reagent (mean ± SD per well; n = 3 experiments). EFluorescence microscopy in fixed SLEx cells from NIMP-R14-treated C57Bl6 mice injected with M. smegmatis revealed no iNOS (red) in cells with DAPI-stained nuclei (blue). Bars, 20 μm; original magnification ×50.

Discussion

Innate MDSCs, phenotypically characterized by Ly-6C, CD11b, and CD31 cell-surface expression and functionally defined by strong NO-mediated in vitro T cell suppression, were recruited to the dermis during the very early stages following BCG vaccination. These cells did not express CD115 or Ly-6G at the cell surface, suggesting that they were not MOs or neutrophils. MDSCs were recently defined in disease states including overwhelming infection or cancer (14). MDSCs originate in the bone marrow, circulate in the blood, and accumulate in lymphoid tissue after the onset of inflammation. They act as a regulatory feedback mechanism to suppress T cells (16). Mouse MDSCs are phenotypically characterized by a combination of markers, including CD11b and Gr1 (Ly6-C/G), and are functionally defined by their potent NO-mediated suppression of T cells (16). Recently, two subsets have been described in mouse tumors: granulocytic MDSCs that express both Ly-6C and Ly-6G and monocytic MDSCs that only express Ly-6C (34, 35). Both subsets are able to block T cell proliferation by different mechanisms associated with the metabolism of l-arginine (14). iNOS is a potent NO effector for T cell suppression by monocytic MDSCs (34). Whereas MDSCs accumulate in lymphoid tissues, inMDSCs appeared early after BCG injection, first in the blood and skin, where they peaked by day 3. In mice, iNOS and NO production induced by classically activated macrophages is essential for the destruction of mycobacteria (36). However, pathogenic mycobacteria escape this harsh environment by
adapting their genetic program for protection against NO (37), iNOS exclusion from the phagosome (38), and macrophage arginase 1 induction (39). Despite phagocytosis and high iNOS levels, skin inMDSCs were unable to kill BCG or the fast-growing M. smegmatis, showing that mycobacteria were also able to escape the hostile NO environment in inMDSCs.

We found that skin CD11b+Gr1+ cells, constituting the early BCG-induced inflammatory infiltrate, were composed of Ly-6C<sup>int</sup>Ly-6G<sup>−</sup> cells and Ly-6C<sup>−</sup>Ly-6G<sup>+</sup> neutrophils. Despite expressing iNOS and TNF, these cells differed from recently characterized TNF and iNOS-producing DCs (3) by a number of aspects: they did not express CD115 or CD11c, their migration from blood to skin was CCR2 independent, and they exerted suppression instead of activation on T cells. However, in respect to their immature phenotype, we do not exclude that inMDSCs might convert to TNF and iNOS-producing DCs under some circumstances. Unexpectedly, we did not detect the recently described inflammatory CX3CR1<sup>lo</sup> MO population between 2 (data not shown) and 72 h.
after BCG inoculation in the skin. However, migrating CX3CR1+ SkEx cells were observed by day 25 after BCG inoculation (Supplemental Fig. 2), demonstrating that these cells were recruited to skin at later time points. Although we cannot exclude that minute amounts of CX3CR1+Ly-6C+ MOs were present during the early stages of infection in skin, the BCG-induced environment may delay the recruitment of inflammatory MOs. Consistent with this, Rotta et al. (40) described that strong inflammatory signals, delivered through TLR4 from endotoxin or Salmonella typhimurium injection in skin, blocked the migration of inflammatory MOs migration and differentiation of DCs in the draining lymph node, occurring under mild inflammatory conditions (10). Upon disruption of homeostasis, the content of the inflammatory cell infiltrate must be tightly regulated. It is widely accepted that neutrophils infiltrate the lesion first and are swiftly followed by MOs, although the role played by neutrophils in the control of MO recruitment is unclear (26, 27, 41). The poor specificity of anti-Gr1 (Ly-6C/Ly-6G) Ab has contributed to confusion in the field. Upon BCG injection, we observed coordinated fluctuations in neutrophil and inMDSC levels in skin infiltrates over time; although neutrophils remained the predominant cell type, inMDSCs numbers increased at later time points. Moreover, the specific depletion of neutrophils markedly increased the recruitment of inMDSCs. These data suggested that the recruitment of inMDSCs was suppressed by neutrophils. The recruitment of neutrophils and inMDSCs to skin was regulated by different signaling pathways. Whereas neutrophils did not need MyD88 to reach the skin, inMDSCs were strictly dependent on MyD88 signaling, highlighting differences between the host mechanisms controlling these two cell populations. MyD88-deficient mice cannot suppress lung Mycobacterium tuberculosis (28, 29) or BCG (42) multiplication. Tissue infiltrates in these mice also display higher levels of Ly-6G+ granulocytic cells than those in MyD88+/+ mice (28), consistent with the MyD88-independent recruitment of neutrophils in response to mycobacteria. Given the suppressive function of inMDSCs, the extensive lung inflammation observed in mycobacteria-infected neutrophils influx and/or the loss of inMDSCs. MyD88 signaling is at the crossroads of TLR-, IL-1R-, and IL-18R-dependent pathways. We did not observe the impaired influx of inMDSCs in skin in TLR 2 × 4/−, TLR 9/−, or IL-1R−/− mice. The receptor upstream from MyD88 involved in the recruitment of these cells remains unknown. However, levels of inMDSC-induced iNOS and NO production in IL-1R−/− mice, which, similarly to MyD88−/− mice (31), are susceptible to tuberculosis infection, were greatly reduced. The β form of pro-IL-1–induced gastric inflammation and cancer have recently been linked to the recruitment of mouse MDSCs (43). Hence, the β form of pro-IL-1 and IL-1R appear to be master regulators of inMDSC activation.

NO produced by inMDSCs was unable to inhibit mycobacterial growth, but completely suppressed T cell proliferation in vitro and curtailed T cell priming in vivo. In BCG-injected mice, increased recruitment of iNOS+ cells to the lymph node after neutrophil depletion correlated with decreased T cell priming. Conversely, in vivo treatment with ATRA, which eliminates MDSCs, increased T cell priming (33). In mice, treatment with ATRA significantly enhanced cancer vaccine efficacy (44). In mycobacteria-induced experimental autoimmune encephalomyelitis models, CD11b+ Gr1+Ly-6G+ cells are compartmentalized in spleen and prevent T cell expansion by secreting NO upon contact with Ag-activated T cells. NO-mediated suppression is counterbalanced by O2− produced by neutrophils (45). These feedback mechanisms have major effects on experimental autoimmune encephalomyelitis pathogenesis. Our data are consistent with the hypothesis that the coordinated innate influx of neutrophils and inMDSCs, and the associated NO/O2− balance, regulates BCG Ag-specific T cell priming during vaccination. This mechanism may control the BCG-induced T cell response level and avoid excessive tissue inflammation. Alternatively, the recruitment of propathogenic cells, such as inMDSCs, which are unable to kill BCG and delay T cell priming, could be an additional mechanism employed by slow-growing mycobacteria to parasitize their host.

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Disclosures

The authors have no financial conflicts of interest.

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Supplementary Figure S1: Comparison of PMN depletion after injection with antibodies NIMP-R14 and 1A8. CX3CR1+/GFP mice were injected i.p. with 1A8 or NIMP-R14 and BCG vaccinated in the ear dermis. Cells migrating from ear skin explants were examined by flow cytometry 72h later, after anti-CD11b, Ly-6C and Ly-6G staining. Both 1A8 and NIMP-R14 treatment induced complete PMN (Ly-6C+ Ly-6G+) depletion and similar Ly-6C+ Ly-6G- cell recruitment. Antibody NIMP-R14 was then used throughout the study.

Martino et al., Fig S1
Supplementary Figure S2: Analysis of CX3CR1 expression on cells migrating from skin explants 25 days after BCG vaccination. CX3CR1^+/GFP^ mice were BCG vaccinated in the ear dermis and cells migrating from ear skin explants were examined by flow cytometry 25 days later, after anti-CD11b, Ly-6C and Ly-6G staining. Ly-6C^+ Ly-6G^- cells (R2) represented 1/3 of the CD11b^+ migrating cells (R1-21%). Among them, 43% were positive for the CX3CR1 monocyte marker.

Martino et al., Fig S2
Supplementary Figure S3: Control of *M. smegmatis* multiplication by classically activated macrophages. Bone-marrow-derived macrophages (4.5 x 10^5 cells/well) activated for 24 h with TNF-α (10 ng/ml) and IFN-γ (100 U/ml) were infected with 4 x 10^5 *M. smegmatis* CFUs. Wells were untreated or treated with the NO inhibitor LMMA (10 μg/ml). Four hours later, cells were washed. Intracellular *M. smegmatis* were determined by plating diluted cell homogenates on agar medium at 4, 24 and 48 h post-infection (Mean +/-SD CFUs per well, n= 6 experiments, Mann Whitney test)

Martino et al., Fig S3
Supplementary Figure S4: IFN-γ secretion by DC-stimulated T cells is abrogated by MDSCs. IFN-γ secretion by BALB/c CD3+ T-cells (10^5/well) stimulated by 10^5 to 2.5x10^4 fixed isogenic BCG-activated DCs (closed squares) was measured by ELISA. C57/BL6 CD11b+ Ly-6C^{int} Ly-6G^{+} SkEx cells (4x10^3 cells) obtained after in vivo PMN depletion (NIMP-R14 treatment, open diamonds) completely suppressed IFN-γ secretion even at the highest DC-T ratio, as did in vitro PMN depleted SkEx cells (open circles). L-NMMA addition to CD11b+ Ly-6C^{int} Ly-6G^{+} cells from in vivo (closed diamonds) or in vitro (closed circles) PMN depleted SkEx cells restored IFN-γ secretion.

Martino et al., Fig S4