Gr-1<sup>d</sup>dimCD11b<sup>+<</sup> Immature Myeloid-Derived Suppressor Cells but Not Neutrophils Are Markers of Lethal Tuberculosis Infection in Mice


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Tuberculosis (TB) disease may progress at different rates and have different outcomes. Neutrophils have been implicated in TB progression; however, data on their role during TB are controversial. In this study, we show that in mice, TB progression is associated with the accumulation of cells that express neutrophilic markers Gr-1 and Ly-6G but do not belong to conventional neutrophils. The cells exhibit unsegmented nuclei, have Gr-1\textsuperscript{dim}Ly-6G\textsuperscript{dim}CD11b\textsuperscript{+} phenotype, and express F4/80, CD49d, Ly-6C, CD115, and CD135 markers characteristic not of neutrophils but of immature myeloid cells. The cells accumulate in the lungs, bone marrow, spleen, and blood at the advanced (prelethal) stage of Mycobacterium tuberculosis infection and represent a heterogeneous population of myeloid cells at different stages of their differentiation. The accumulation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells is accompanied by the disappearance of conventional neutrophils (Gr-1\textsuperscript{hi}Ly-6G\textsuperscript{hi}–expressing cells). The Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells suppress T cell proliferation and IFN-\gamma production in vitro via NO-dependent mechanisms, that is, they exhibit characteristics of myeloid-derived suppressor cells. These results document the generation of myeloid-derived suppressor cells during TB, suggesting their role in TB pathogenesis, and arguing that neutrophils do not contribute to TB pathology at the advanced disease stage.

Immune reactions play both protective and pathological roles during tuberculosis (TB). Immunological mechanisms mediating TB protection have been studied in details. In contrast, mechanisms driving TB progression remain poorly understood.

One of the mechanisms that have been implicated in TB pathology and progression is neutrophilic inflammation. Indeed, neutrophils are known to induce tissue damage. In humans, high numbers of neutrophils were found in bronchoalveolar lavage fluids of patients with severe TB disease and lung tissue cavitation (1–3). In mice, cells expressing neutrophilic markers Gr-1 and/or Ly-6G accumulated abundantly in the lungs of susceptible animals; in several studies, depletion of Gr-1–expressing cells ameliorated TB outcome (3–6). In contrast, a large number of observations support the role for neutrophils in TB protection: these cells can phagocytize mycobacteria, produce numerous bactericidal molecules, arm macrophages to kill mycobacteria and promote initiation of T cell immune responses (7–10). An association between low neutrophilic numbers, low plasma levels of neutrophil-derived bactericidal peptides, and high risk of TB development has been demonstrated (11, 12), further supporting the impact of these cells in TB protection.

To address the role for neutrophils during TB, we have recently evaluated their response in (A/Sn × I/St)F2 hybrids originating from TB-resistant A/JSnYCit (A/Sn) and TB-susceptible I/StYCit (I/St) mice. Following Mycobacterium tuberculosis challenge, F2 mice exhibited different rates of TB progression (13). F2 mice that succumbed rapidly to M. tuberculosis infection had extremely high numbers of Gr-1– and Ly-6G–expressing cells in their lungs. Gr-1/Ly-6G–expressing cells are usually considered as neutrophils. However, neutrophils express Gr-1/Ly-6G markers at high levels (14). The cells accumulating in the lungs of TB-susceptible F2 mice in our study expressed Gr-1\textsuperscript{dim}/Ly-6G\textsuperscript{dim} phenotype. The nature of these cells and their relationship to neutrophils were unclear.

Although almost nothing was known about Gr-1\textsuperscript{dim}/Ly-6G\textsuperscript{dim} cells during TB, similar cells had been described and characterized in detail during other pathological conditions, in particular, during tumor progression (15, 16). The cells expressed a common myeloid marker CD11b (phenotype Gr-1\textsuperscript{dim}CD11b\textsuperscript{+}), inhibited T cell responses and were therefore referred to as myeloid-derived suppressor cells (MDSCs). MDSCs represent a heterogeneous population of myeloid cells found at different stages of their differentiation. Two main subsets of MDSCs, monocytic and granulocytic, have been described according to their phenotype (Gr-1\textsuperscript{dim}CD11b\textsuperscript{+}Ly-6G\textsuperscript{dim}F4/80\textsuperscript{dim}CD49d\textsuperscript{hi} or Gr-1\textsuperscript{dim}CD11b\textsuperscript{+}Ly-6G\textsuperscript{dim}F4/80\textsuperscript{+}CD115\textsuperscript{+}CD49d\textsuperscript{−}, respectively) and nuclear morphology (15–17). MDSCs are rare in steady-state conditions, but they accumulate abundantly during different pathologies (especially those inducing hyperinflammatory response) and contribute to their progression (18–23).

We hypothesized that Gr-1\textsuperscript{dim} cells, identified in our previous studies, represented MDSCs (24). In this study, we present evidence supporting this hypothesis. We demonstrate that Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} MDSCs
cells: 1) accumulate at advanced stage of TB infection in mice of different strains; 2) exhibit characteristics of immature myeloid cells; 3) suppress T cell responses; and 4) are rather associated with TB progression and lethality than mature neutrophils. To our knowledge, this is the first description of MDSCs and their role during TB.

Materials and Methods

Mice

Inbred female I/St, A/Sn, and C57BL/6(C3H)F1 (B6) mice 2–3 mo of age were used (19–21 g). All mice were bred in the Animal Care Facility at the Central Tuberculosis Research Institute (Moscow, Russia) in accordance with Russian Ministry of Health Guideline number 755 and the U.S. National Institutes of Health Office of Laboratory Animal Welfare Assurance number A5502-01. Water and food were provided ad libitum. All experimental procedures were approved by the Central Tuberculosis Research Institute Institutional Animal Care and Use Committee.

Bacteria and infection

In most experiments, mice were infected intratracheally (i.t.) with $2.3 \times 10^4$ CFUs/mouse of midlog-phase M. tuberculosis strain H37Rv Pasteur as described previously (25). To monitor disease progression, mice were weighed before the challenge and then every 3–7 d. If not indicated otherwise, experiments were performed on days 17 and 24 postinfection. Uninfected mice were included in all experiments (“day 0”). In some experiments, mice were infected with $10^3$ CFUs using an Inhalation Exposure System (Glas-Col, Terre Haute, IN) and analyzed on days 22, 80, 160, and 210. Mycobacterial loads were assessed by preparing homogenates of the lungs (upper right lobe) or suspensions of spleen and bone marrow (BM) cells and plating 0.1 ml of their serial 10-fold dilutions onto Dubos Agar; colonies were enumerated after 18–20 d.

Flow cytometry

Suspensions of lung cells were prepared using an enzyme digestion method (26). Lungs were perfused with 0.02% EDTA-PBS to wash blood vessels and incubated in RPMI 1640 medium containing collagenase/DNase I, and cell suspensions were washed. Spleen cell (SC) suspensions were prepared by mild homogenization. To obtain BM cells, tibia and femurs were flushed with 199 medium containing 1% FCS and 10 mM HEPES. Cells were treated with anti-CD16/CD32 mAbs (eBioscience, San Diego, CA) and stained with different combinations of the following Abs: FITC-anti-CD4, PE-anti–Gr-1 (clone RB6-8C5), and APC-anti-CD11b (all obtained from eBioscience); FITC-anti–Ly-6G (clone 1A8) and PerCP-anti-CD4 (BD Pharmingen, San Diego, CA); PerCP-anti–Ly-6C (clone HK1.4) and biotin-anti-CD49d, followed by PerCP-streptavidin and APC-anti-CD11b (all obtained from BioLegend, San Diego, CA). In preliminary experiments, cells were also stained with Live/Dead Fixable Far Red Dead Cell Stain Kit (Life Technologies, Carlsbad, CA). The experiments confirmed that Gr-1dimCD11b+ cells accumulating in the lungs and BM at the advanced stage of infection were alive. Cells were analyzed using a BD Biosciences FACSort or Canto II with CellQuest Pro, BD FACSDiva 7 (BD Biosciences), and FlowJo (Tree Star) software. In most experiments, at least 40,000 events were analyzed.

Magnetic cell sorting

Suspensions of BM cells were enriched for Gr-1dimCD11b+ and Gr-1hiCD11b+ populations using two-step magnetic cell sorting. Briefly, BM cells were stained with PE-anti–Gr-1 mAbs (eBioscience), incubated with anti-PE MultiSortMicrobeads (Miltenyi Biotec, San Diego, CA), washed, and separated by sequential passage through LS MACS column (Miltenyi Biotec). The positive fraction was enriched for Gr-1hiCD11b+ cells (purity, 60–75%; all CD11b+; Fig. 3A). The negative fraction was composed of Gr-1dimCD11b+ and Gr-1ermCD11b+ cells. For the isolation of Gr-1ermCD11b+ cells, the negative fraction was incubated with APC-anti-CD11b mAbs, magnetically labeled with anti-APC MultiSort Microbeads (Miltenyi Biotec), and sorted on an LS MACS column to collect the positive fraction. The later contained mostly Gr-1ermCD11b+ cells (purity, 90–95%; Fig. 3A).

Suppression assays

SC were isolated from the spleens of uninfected mice, labeled with CFDA-SE (1–10 μM, 10 min, room temperature), washed with PBS containing 10% FCS, and cultured in 24- or 96-well plates (9 × 10^5 cells/ml) covered with immobilized anti-CD3 mAbs (1 μg/ml). Suppressor cells were added to the cultures at 1:1 ratio. Suppressors were BM cells either unseparated or enriched for Gr-1ermCD11b+ or Gr-1CD11b+ populations. Four days later, cells were harvested and stained with PE-anti–CD4 and PerCP-anti-CD8 mAbs, and proliferation of CD4+ and CD8+ cells was assessed by flow cytometry using CFSE dilution assay. IFN-γ production was measured in culture supernatants using Mouse IFN-γ ELISA Ready-SET-Go! (eBioscience). In some experiments, SC were stimulated with immobilized anti-CD3 mAb, and percentages of IFN-γ-producing cells were determined using intracellular cytokine staining (27).

To test the ability of BM cells to suppress M. tuberculosis–specific responses, I/St mice were immunized with a CFA (100 μg given s.c. into the footpads). Ten days later, popliteal lymph node (LN) cells were isolated and cultured in vitro in the presence of M. tuberculosis sonicate (10 μg/ml) and BM cells. Proliferation of LN cells was assessed using [3H]thymidine incorporation and CFSE dilution assays.

In inhibition experiments, SC and BM cells were cocultured in the presence of one of the following inhibitors: 500 μM N-hydroxy-L-arginine (l-NHOA; Merck, Darmstadt, Germany), 100 μM Nω-monomethyl-L-arginine (l-NMMA; Calbiochem, San Diego, CA), and 1000 U/ml catalase (Sigma-Aldrich, St. Louis, MO). In Transwell experiments, SC were stimulated with anti-CD3 at the bottom of Transwells, and BM cells were placed in 3-μm Transwell chambers (Costar-Corning, Amsterdam, The Netherlands).

Adoptive cell transfer

To test the ability of Gr-1ermCD11b+ cells to inhibit T cell responses in vivo, adoptive transfer experiments were performed. Donor mice were challenged with M. tuberculosis i.t. On day 24 postchallenge, BM cells were isolated and enriched for Gr-1ermCD11b+ population. The cells were transferred into recipient mice (2 × 10^6/mouse) that had been challenged with M. tuberculosis either i.t. (17 d prior the transfer) or aerogenically (74 d prior to the transfer). Three days after the first transfer (i.e., on days 20 or 77, respectively), recipient mice received the second transfer of Gr-1ermCD11b+ cells (the later were freshly isolated from another group of donor mice, challenged 24 d prior to the experiment). Plating of Gr-1ermCD11b+ cells showed that the inoculum contained <100 CFUs (i.e., <0.1% of M. tuberculosis present in the lungs of recipient mice). Plating of 100 CFUs into recipient mice did not add to their M. tuberculosis burden (i.e., the contamination of Gr-1ermCD11b+ cells with M. tuberculosis did not affect recipient M. tuberculosis burden significantly). Three days after the second transfer, recipient mice were sacrificed, and the levels of IFN-γ production in the lungs were analyzed using ELISA. The time points for cell transfers were chosen based on the following: 1) antimycobacterial T cell responses are known to set up after the second week of infection (28); 2) in i.t. challenge model, recipient mice died by the end of week 4 postchallenge; and 3) in aerosol challenge model, on days 74–80, mice were at the stable stage of infection.

Statistical analysis

Data are shown as mean ± SEM. Differences between the means of experimental groups were analyzed using the nonparametric Mann–Whitney U test. Differences were considered significant where $p < 0.05$. The correlation between body weight loss and mean fluorescence intensity (MFI) of Gr-1 was assessed using nonparametric Spearman correlation analysis (GraphPad Software, San Diego, CA).

Results

Gr-1ermCD11b+ cells accumulated in the lungs, spleen, BM, and blood of mice at the advanced stage of TB infection

We first sought to characterize the kinetics of Gr-1erm response following M. tuberculosis challenge. Therefore, we challenged TB-susceptible I/St mice with M. tuberculosis given i.t. and followed TB progression and the frequencies and the numbers of Gr-1ermCD11b+ cells in different organs. TB progression was evaluated by determining postinfection body weight loss, a vital indicator of TB severity. All mice gained weight during first 2 wk postchallenge (“stable stage” of infection), started to undergo wasting after day 17 (“initiation of disease progression”), and lost 15 ± 2% of their initial weight by day 24 (prelethal stage of disease; Fig. 1A). CFU numbers in the lungs increased 50-fold since day 17 until day 24 (Fig. 1B). If not sacrificed, mice died by day 26–28 postchallenge.
In uninfected mice, Gr-1dimCD11b+ cells were largely absent from the lungs, spleen, and blood (< 4%) and were also rare in the BM (< 8%; Fig. 1C–E; data not shown). The frequencies and the numbers of Gr-1dimCD11b+ cells slightly increased in the lungs by day 17 (2- to 3-fold; \( p < 0.05 \)) and dramatically increased in all organs by day 24 postchallenge (7- to 40-fold in the lungs and spleen and 3- to 10-fold in the BM and blood, \( p < 0.01; \) Fig. 1D, 1E; data not shown). As Gr-1dimCD11b+ cells accumulated, the frequencies and the numbers of Gr-1hiCD11b+ cells decreased. It should be noted that cells, which in infected mice fall into the “Gr-1hi” gate, had a lower expression of Gr-1 compared with Gr-1hi cells from uninfected mice; moreover, in infected mice, Gr-1hi cells represented rather an extent of Gr-1dim population than true Gr-1hi cells present in the BM of uninfected mice, Gr-1hi cells expressed markers of neutrophils, whereas Gr-1dimCD11b+ cells coexpressed neutrophilic and monocytic markers. These differences were seen in all analyzed organs (Fig. 2; data not shown).

Following the infection, true Gr-1hiCD11b+ cells disappeared: all Gr-1-expressing cells appeared as one large scattered population that represented a continuum of cells with different levels of Gr-1 expression (Figs. 1C, 2A). Previously, several authors have demonstrated that the levels of Gr-1 and Ly-6G expression are indicative of a degree of granulocytic cell differentiation and maturation (14, 30). Therefore, we compared the phenotype of CD11b+ that in infected mice expressed Gr-1 at different levels (i.e., Gr-1dim and Gr-1hi cells) (Fig. 2A). Compared with Gr-1hi cells, Gr-1dim cells had a lower expression of Ly-6G and a higher expression of F4-80 and CD49d (Fig. 2D, 2F). Gr-1-expressing cells derived from the lung tissue had similar expression profiles (Fig. 2E).

These data indicated that, at the advanced stage of the disease, Gr-1-expressing cells were less differentiated. Therefore, we analyzed the expression of CD117 (c-kit) and CD135 (Flt-3) on Gr-1dimCD11b+ cells. In uninfected mice, CD117- and CD135-expressing cells had a lower expression of Gr-1 and Ly-6G, and a higher expression of F4-80 and CD49d (Fig. 2D, 2F). Gr-1-expressing cells derived from the lung tissue had similar expression profiles (Fig. 2E).

In contrast, all Gr-1dimCD11b+ and all Gr-1hiCD11b+ cells were positive for Ly-6C (Fig. 2C–E).

To further characterize Gr-1dimCD11b+ population, we isolated BM cells from M. tuberculosis–infected mice, enriched them with Gr-1hiCD11b+ and Gr-1dimCD11b+ populations and analyzed their nuclear morphology using light and confocal microscopy (Fig. 3). The
majority of Gr-1hiCD11b+ cells had either segmented nuclei (>20% of cells) or constricted ring-shaped nuclei with a wide cytoplasmic center (larger than the width of the ring, >50% of cells; Fig. 3B, 3D) (i.e., exhibited characteristics of polymorphonuclear cell (PMN)-like cells) (31). In contrast, Gr-1dimCD11b+ population was heterogenic and largely composed of MNC-type cells and myelo/monocytic precursors: the cells had bean-shaped nuclei, ring-shaped nuclei with a small cytoplasmic center (the cytoplasmic center of the ring smaller than the width of the ring, a feature characteristic for mononuclear cells (31) or closed nuclei (Fig. 3C, E)). The percentage of PMN-like cells in Gr-1dimCD11b+ population was small (<10%).

Thus, in both phenotypic and morphological analyses, Gr-1dimCD11b+ cells appeared as a heterogeneous population of immature myeloid cells, largely of MNC type. Gr-1dimCD11b+ cells exhibited suppressor activity and inhibited T cell proliferation and IFN-γ production

We then tested whether Gr-1dimCD11b+ cells were similar to MDSCs and could suppress T cell responses. As a source of Gr-1dimCD11b+ cells, we used BM cells. The usage of lung cells was difficult, because their magnetic sorting was of low efficiency, and fluorescence-activated sorting of M. tuberculosis–infected lung cells was unavailable.

In the first experimental setting, total populations of BM cells were tested for their suppressive activity. BM cells were isolated from uninfected (BMd0) or M. tuberculosis–infected mice on day 17 ("BMd17") or 24 ("BMd24") postinfection. The cells were added to naive SC stimulated polyclonally with anti-CD3 mAbs, and proliferation of CD4+ and CD8+ lymphocytes was assessed using CFSE dilution assay (Fig. 4A). BMd0 did not inhibit proliferation of CD4+ T cells; BMd17 and BMd24 inhibited proliferation by 38 ± 12 and 85 ± 10%, respectively (Fig. 4A, 4B). Similar results were obtained when proliferation of CD8+ cells was assessed (data not shown). Besides proliferation, BMd24 also blocked IFN-γ production, as evidenced by a decrease in the concentrations of IFN-γ in culture supernatants (Fig. 4C) and in the frequencies of IFN-γ–producing cells (data not shown). Separation of SC and BM cells by a Transwell membrane abrogated suppression (Fig. 4D), indicating
that direct contacts between suppressor and responding cells or their close colocalization were necessary for successful suppression.

In a separate experiment we tested whether BM cells were able to suppress T cell response to *M. tuberculosis*–specific Ags. Mice were immunized with CFA; LN cells were isolated and stimulated in vitro with *M. tuberculosis* sonicate in the presence or absence of BMd24 cells. In the presence of BMd24 cells, a complete inhibition of cell proliferation was registered ([3H]thymidine incorporation and CFSE dilution assays) (Fig. 4J; data not shown).

We next examined whether suppressor activity of BM cells was mediated by Gr-1dimCD11b+ population (as we supposed). For that, we enriched BM cells with Gr-1dimCD11b+ cells using magnetic cell sorting and tested their ability to suppress SC proliferation and IFN-γ production. Gr-1dimCD11b+ cells isolated either from uninfected or *M. tuberculosis*–infected mice (days 0 and 24, respectively) inhibited T cell proliferation and IFN-γ production by 70–80% (Fig. 4E, 4F). Simultaneously with Gr-1dimCD11b+ cells, we also isolated Gr-1hiCD11b+ cells. These cells exhibited suppressor activity mainly when isolated from *M. tuberculosis*–infected mice (day 24 postchallenge; Fig. 4E, 4F).

To summarize, Gr-1dimCD11b+ cells exhibited suppressor activity when isolated from the BM of either uninfected or *M. tuberculosis*–infected mice. In contrast, total population of BM cells and Gr-1hiCD11b+ cells acquired suppressor activity following infection. Of note, in infected mice (day 24), BM contained 6-fold more Gr-1dimCD11b+ cells and 3-fold less Gr-1hiCD11b+ cells than in control mice. Furthermore, as discussed above, Gr-1hiCD11b+ cells were more monocytic. Thus, our observations suggested that suppressor activity was the intrinsic property of immature (Gr-1dim) myeloid cells and that these cells, rather than neutrophils, were responsible for BM-mediated suppression of T cell responses.

*Gr-1dimCD11b+ cells used NO to suppress T cell proliferation and function*

It has been reported that suppression of T cell responses by MDSCs may be mediated by different mechanisms, including iNOS–dependent production of NO, arginase I–dependent depletion of arginine from the environment of T cells, and generation of reactive oxygen intermediate (32). We investigated whether inhibition of these pathways altered immunosuppression mediated by Gr-1dimCD11b+ cells.

The addition of either l-NOHA, an inhibitor of arginase, or catalase, reactive oxygen intermediate eliminator, did not alter the suppression. In contrast, l-NMMA, an inhibitor of iNOS, almost completely abrogated the suppression and restored T cell proliferation and IFN-γ production in all suppressed cultures, suggesting that the suppression was NO mediated (Fig. 4G, 4H; data not shown).

A role for NO in the suppression of T cell responses was further confirmed by high concentrations of nitrite in all cultures where suppression was detected (i.e., in the cultures of SC and Gr-1dimCD11b+ cells, BMd24, or Gr-1hiCD11b+ cells isolated from BMd24) and by low nitrite concentrations in all the cultures where suppression was absent, either initially (cultures containing SC and BMd0) or Gr-1dimCD11b+ cells isolated from BMd0) or because of its inhibition (cultures containing SC, “suppressive” BM cells and l-NMMA, Fig. 4I and data not shown).

Altogether, in our model, the suppression was mediated mainly by NO, the mechanism of suppression characteristic of monocytic population of MDSCs (33, 34).

*Accumulation of Gr-1dimCD11b+ cells is a characteristic feature of severe TB disease in mice of different strains*

The studies described above were performed in I/St mice, highly susceptible to *Mtb* infection (26, 35). We next examined whether in A/Sn and B6 mice genetically more resistant to *Mtb* infection (26, 35, 36), TB progression was also accompanied by the generation and the accumulation of Gr-1dimCD11b+ cells.

Similarly to I/St mice, in uninfected A/Sn mice, Gr-1dimCD11b+ cells were rare. On day 24 postchallenge, the frequencies and the numbers of Gr-1dimCD11b+ cells increased in I/St mice 5- to 20-fold, whereas in A/Sn mice, only 2- to 6-fold (Fig. 5A). Of note, I/St mice died by days 26–28 postchallenge, whereas A/Sn mice survived until days 33–35. On day 31, A/Sn mice exhibited severe TB disease, characterized by high concentrations of nitrite in all cultures and a complete inhibition of cell proliferation (Fig. 4G; data not shown). We observed similar trends in B6 mice (data not shown).
significant wasting and a pronounced increase in Gr-1dimCD11b+ cells in the lungs (33-fold) and BM (8-fold). B6 mice started exhibiting significant wasting by day 26. At this time point, the frequencies and the numbers of Gr-1dimCD11b+ cells in their lungs, BM, and spleen also increased (Fig. 5B–D).

In the experiments described above, the rate of TB progression in susceptible I/St and resistant B6 mice differed only slightly. This was likely due to a relatively high dose of bacteria used for the infection (2.3 ± 10^3 CFU/mouse given i.t.). We next addressed whether a low-dose challenge would also lead to the accumulation of Gr-1dimCD11b+ cells. For that, I/St and B6 mice were challenged with 10^2 CFUs of *M. tuberculosis* given aerogenically.

During the stable stage of infection (days 22, 80, and 160) Gr-1dimCD11b+ cells were rare, the percentages of Gr-1hiCD11b+ remained stable in all mice (Fig. 5F, 5G); mice did not exhibit wasting, although *M. tuberculosis* loads progressively increased (Fig. 5H). By day 210, B6 mice did not waste significantly, while I/St mice exhibited significant (>15%) wasting, suggesting they reached the prelethal stage of disease. All mice were sacrificed for the analysis. In B6 mice, the frequencies of Gr-1dimCD11b+ and Gr-1hiCD11b+ cells did not change significantly (Fig. 5G). In I/St mice, the frequencies of Gr-1dimCD11b+ cells increased (5-fold); Gr-1hiCD11b+ cells disappeared (Fig. 5F). *M. tuberculosis* burden increased in both B6 and I/St mice (compared with day 80), although to a different degree (B6 mice: 18-fold in the lungs and 3-fold in the BM; I/St mice: 200-fold in the lungs and 12-fold in the BM) (Fig. 5H). Phenotypic analysis of Gr-1dimCD11b+ cells accumulating during chronic infection showed that they expressed phenotype similar to Gr-1dimCD11b+ cells found in acutely infected mice. Functional analysis revealed their ability to suppress
FIGURE 5. Gr-1dimCD11b+ cells accumulate in TB-susceptible and TB-resistant mice at prelethal stage of acute and chronic infection. (A–E) Acute infection; mice were challenged i.t. with 2.3 × 10^3 CFUs. (F–H) Chronic infection; mice were challenged aerogenically with 10^2 CFUs. (A) Kinetics of Gr-1dimCD11b+ cells in the lungs of I/St and A/Sn mice. Percentages indicate body weight loss at the corresponding time points. (B–D) Frequencies of Gr-1dimCD11b+ cells in different organs of I/St, A/Sn, and B6 mice at indicated time points (two experiments; n = 6–10/group). (E and H) *M. tuberculosis* loads in the lungs and BM of mice challenged i.t. (E) or aerogenically (H). (F and G) Frequencies of Gr-1dimCD11b+ and Gr-1hiCD11b+ cells in the BM of I/St (F) and B6 (G) mice following the chronic infection. (I) Correlation between body weight loss and the levels of Gr-1 expression in I/St and A/Sn mice (n = 20).

T cell proliferation in vitro (i.e., the cells could be ascribed to MDSC) (data not shown).

Thus, MDSCs accumulated following both acute and chronic infection when mice reached the advanced stage of disease.

As discussed above, the levels of Ly-6G and Gr-1 expression are indicative of a degree of myeloid cell differentiation and maturation (14, 30). Therefore, we assessed whether in our model there was an association between TB progression and the levels of Gr-1 expression by the whole population of CD11b+ cells. A significant inverse correlation between body weight loss and the levels of Gr-1 expression was found (r > 0.6; p < 0.02; Fig. 5I), supporting an association between TB progression and myeloid cell immaturity.

To summarize, hematopoietic shifts resulting in the generation and the accumulation of immature myeloid cells with suppressor activity were a common trait of progressing TB infection in mice.

Adoptive transfer of Gr-1dimCD11b+ cells

We next addressed whether Gr-1dimCD11b+ cells could mediate immune suppression in vivo. For that, we transferred Gr-1dim CD11b+ cells adoptively. The cells were isolated from the BM of *M. tuberculosis*–infected donor mice challenged i.t. 24 d before the transfer. In the first experimental setting, we addressed how the transfer of Gr-1dimCD11b+ cells affected host immune response and TB progression during the acute infection. Recipient mice were challenged with *M. tuberculosis* given i.t.: on days 17 and 20 postchallenge (i.e., before endogenous Gr-1dimCD11b+ cells started accumulating and mice progressed to fatal disease), Gr-1dim CD11b+ donor cells were transferred into the recipient mice. Compared with control mice, recipients of Gr-1dimCD11b+ cells had significantly lower content of IFN-γ in the lung tissue (p = 0.02; Fig 6A). There was a tendency toward an accelerated wasting of recipient mice (p = 0.06; Fig. 6C); but *M. tuberculosis* burden in the lungs, spleen, and BM did not change (p > 0.6; Fig. 6B; data not shown).

In the second experimental setting, we examined whether Gr-1dimCD11b+ cells could affect immune response during the stable phase of the chronic infection. Recipient mice were infected aerogenically with a low dose of *M. tuberculosis*; Gr-1dimCD11b+ cells were transferred on days 74 and 77 postchallenge, and mice were analyzed on day 80. We saw no significant changes in IFN-γ production in the lungs of recipient mice (p = 0.34; Fig. 6A); the transfer did not affect mice weight and *M. tuberculosis* burden (Fig. 6B, 6D). Thus, in vivo Gr-1dimCD11b+ cells suppressed IFN-γ response at the advanced stage of the acute TB infection but did not exhibit evident suppressor activity during the stable stage of the chronic infection.

There are several possible explanations for these discrepancies. First, it is possible that the effect of cell transfer depended on the ability of the host to counteract the suppression: such ability could be more efficient during the stable stage of infection but could go out of control at the advanced stage of disease. Second, it is possible that Gr-1dimCD11b+ cells acquired suppressor activity when transferred into the acutely infected recipients but failed to do so when transferred into the chronically infected mice. Indeed, it is

FIGURE 6. Effect of Gr-1dimCD11b+ cells on IFN-γ production in vivo. Recipient mice were challenged i.t. [2.3 × 10^3 CFU/mouse, (A)–(C)] or aerogenically [10^2 CFU/mouse, (A), (B), (D)] and transferred with Gr-1dimCD11b+ cells. (A and B) IFN-γ production (A) and *M. tuberculosis* loads (B) in the lungs of recipient mice (n = 3–11/group). (C and D) Weight loss of recipient mice. Arrows indicate days of cell transfer.
known that MDSCs need to be activated to acquire suppressive activity, and IFN-γ is one of their most potent activators (21, 37). As shown in Fig. 6A, in the acutely infected recipients, the levels of IFN-γ production were higher (control groups of mice; Fig. 6A), which could account for a higher suppressive activity of Gr-1dimCD11b+ cells in these recipients.

Irrespective of whether Gr-1dimCD11b+ cells were able to suppress immune response in vivo or not, to our knowledge, our results for the first time demonstrate that Gr-1dimCD11b+ cells accumulating during progressing M. tuberculosis infection do not belong to typical neutrophils but rather represent a population of immature myeloid cells. The accumulation of these cells occurs as a result of hematopoietic shifts, because it is accompanied by the disappearance of neutrophils and is observed in different organs, including the BM. Hematopoietic shifts described in our study represent a common trait of fatal TB in mice, and potentially may represent a new pathogenic factor of TB progression and severity in human.

**Discussion**

**Gr-1dimCD11b+ cells and neutrophils during M. tuberculosis infection**

In this study we have demonstrated that progression of M. tuberculosis infection is tightly associated with the generation and the accumulation of Gr-1dimCD11b+ cells in the lungs, BM, spleen, and blood of infected mice. Phenotypic, morphological, and functional analyses of Gr-1dimCD11b+ cells characterized them as immature myeloid suppressor cells: 1) the cells coexpressed neutrophilic (Gr-1 and Ly-6G) and monocytic (F4/80, CD49d) markers; 2) the cells had nuclei of immature monocytes/granulocytes and myelomonocytic precursors; 3) a subset of Gr-1dimCD11b+ cells expressed CD117 and CD135; and 4) the cells were able to suppress T cell responses in vitro. To the best of our knowledge, this is the first description of MDSCs during TB.

During other pathological conditions, two major subsets of MDSCs, monocytic and granulocytic have been described previously (16). Gr-1dimCD11b cells described in our study likely belonged to the monocytic lineage and contained cells that were at different stages of their differentiation. This is supported by cell phenotype, nuclear morphology, and NO-mediated suppression (feature characteristic for monocytic population of MDSCs).

Previously, several studies have described the accumulation of Gr-1–expressing cells during severe TB infection and have associated the accumulation of these cells with TB susceptibility and severity (4–6). However, most of the studies did not take into account the levels of Gr-1 expression and considered all Gr-1–expressing cells as neutrophils. Our study demonstrates that Gr-1–expressing cells that accumulate abundantly at the late stages of M. tuberculosis infection differ from typical neutrophils: in our model, the cells expressed Gr-1 and Ly-6G at low levels and represented a mixture of myeloid cells that were at different stages of their differentiation/maturity. Moreover, the accumulation of Gr-1dimCD11b+ cells was accompanied by the disappearance of conventional neutrophils (Gr-1hiCD11b+ cells), indicating on severe hematopoietic shifts that took place at the advanced stage of TB disease. Of note, the more severe was TB disease, the lower were the levels of Gr-1/Ly6G expression on CD11b+ cells (i.e., the lower was their maturity) (Fig. 5f). Thus, hematopoietic shifts resulting in the accumulation of immature myeloid cells and gradual disappearance of neutrophils was a characteristic trait of advanced TB infection in mice.

Our data on immaturity of Gr-1dimCD11b+ cells accumulating during severe TB infection can help explain some of conflicting data on the role for neutrophils in TB pathogenesis. Indeed, on the one hand, neutrophils were shown to phagocyte and kill mycobacteria, produce microbial peptides, activate macrophages for M. tuberculosis killing, and stimulate initiation of antimycobacterial T cell responses (8–10, 38). In high TB-burden settings, low counts of neutrophils and low plasma levels of neutrophil-derived microbial peptides were associated with high risk of TB development (11, 12), suggesting a significant role for neutrophils in TB protection. On the other hand, we and others have previously demonstrated that neutrophils (i.e., Gr-1–expressing cells) accumulate abundantly in the lungs of mice susceptible to M. tuberculosis infection (5, 6); in some studies, neutralizing anti-Gr-1 Abs were shown to ameliorate the course of TB infection in mice (4, 6). On the basis of these observations, neutrophils were implicated in TB progression and pathology (3).

Results obtained in our study demonstrate that Gr-1–expressing cells accumulating during severe TB do not necessarily represent neutrophils. Thus, conclusions on the pathological role for neutrophils made in experimental studies, in which neutrophils were identified (or depleted) based on their Gr-1/Ly-6G positivity, should be revised to take into account the fact that Gr-1/Ly-6G markers also are expressed by immature myeloid cells. Of note, other authors also noticed atypical features of “neutrophils” accumulating in the mouse lung during severe TB. For example, earlier Keller et al. (6) analyzed Gr-1hi cells in the lungs of TB-susceptible and TB-resistant mice. Although in TB-resistant mice Gr-1+ cells were granulocytic, Gr-1hi cells accumulating in the lungs of susceptible mice had signs of “aberrant differentiation.” This observation corresponds to the results of our study.

Altogether, hematopoietic shifts resulting in the accumulation of immature myeloid cells and gradual disappearance of neutrophils was a characteristic feature of the advanced stage of TB.

**Mechanisms promoting Gr-1dimCD11b+ cell generation during TB**

Results obtained in our study raise several questions. One of the questions is which factors induce Gr-1dimCD11b+ cells during TB.

Studies performed in other pathological conditions (e.g., tumors) suggest that the main factors that induce MDSC generation and activation are growth factors and proinflammatory cytokines (i.e., CSF, GM-CSF, G-CSF IL-1β, IL-6, PGE2, and so on) (32, 39–41). Of note, these factors are abundantly produced during severe TB infection. For example, we have recently demonstrated high expression of IL-1β, IL-6, matrix metalloproteinase 8, and CXCL2 and several other factors in the lungs of (A/Sn×I/St)F2 mice highly susceptible to TB (13). More recently, we found much higher expression of GM-CSF in I/St compared with A/Sn mice (E.N. Tsiganov, E.M. Verbina, and I.V. Lyadova, unpublished observations). Other authors reported overproduction of G-CSF, IL-1β, IL-6, and other inflammation-related factors during lethal TB infection in CARD9 and IL-1R knockout mice (4, 42). Thus, exacerbated inflammation represents a common feature of severe lethal TB infection and likely can serve as a major cause for the accumulation of Gr-1dimCD11b+ cells at the advanced stage of TB.

Among other factors that potentially can switch hematopoiesis to generate Gr-1dimCD11b+ cells are mycobacteria and mycobacteria-derived factors. Indeed, at the prelethal stage of infection (i.e., at the time point when Gr-1dimCD11b+ cells became abundant), M. tuberculosis burden increased significantly in different organs. Moreover, M. tuberculosis could be found in the BM of infected mice. Next, it has been demonstrated that hematopoietic stem cells express TLR, and TLR-mediated signals skew hematopoiesis to myeloid lineage (43). In contrast, in our experimental setting, the accumulation of Gr-1dimCD11b+ cells correlated better...
with weight loss than with *M. tuberculosis* burden; TB severity and lethality are not directly associated with *M. tuberculosis* burden (13, 44). Thus, the role for *M. tuberculosis*–derived signals in hematopoietic shifts and generation MDSCs during TB is yet to be determined.

**Possible roles of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells in TB pathogenesis**

Other interesting questions raised by our data are: what is the role for Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells in TB pathogenesis, that is, does the accumulation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells cause TB progression, witness it or result from it? Why fatal TB is always accompanied by the disappearance of neutrophils, and neutrophils have been documented a strong association between the accumulation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells and TB fatality, the role, which these cells play in TB pathogenesis, is to be determined.

On the basis of our data on the ability of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells to suppress IFN-γ response, the main host protective mechanism, it is logical to suppose that Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells can directly contribute to TB fatality. However, the pathways whereby Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells are involved in TB pathogenesis may be more complicated.

It is now understood that uncontrolled T cell responses are deleterious (45). Thus, Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells may have a regulatory function, and their accumulation at the advanced stage of disease may represent the last attempt of the host to dampen exacerbated immune responses. This concept is supported by the fact that the generation of MDSCs is driven by proinflammatory factors, and their activation is induced by IFN-γ (16). Thus, MDSCs are generated and activated in the circumstances when the host critically needs to balance immune responses.

It is also possible that the generation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells contributes to TB progression indirectly, for example, through the concomitant disappearance of neutrophils. As shown in this study, the accumulation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells is always accompanied by the disappearance of neutrophils, and neutrophils have been implicated in TB protection (see above).

Besides suppressing and/or regulating T cell responses, Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells also may play other roles. For example, they may secrete proinflammatory factors and/or serve as a reservoir for *M. tuberculosis* (our preliminary observations). These functions of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells need further investigations.

Finally, we cannot rule out the possibility that the accumulation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells does not contribute to TB pathogenesis but simply witnesses it. Indeed, proinflammatory factors, the major inductors of MDSCs and TB pathology, are abundantly produced during severe TB infection. The accumulation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells may therefore represent a side effect of overwhelming inflammatory response.

An intriguing question is whether there is an association between the generation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells and TB susceptibility, that is, whether susceptible mice have intrinsically higher propensity to generate Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells compared with more resistant mice. We believe that the generation and the accumulation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells is rather a consequence than an initial cause of TB progression. Initial pathways leading to rapid TB progression are likely different in genetically different hosts. Such pathways may include poor ability of the host to restrict *M. tuberculosis* growth; overwhelming inflammatory response to mycobacteria, for example, because of inflammatory hyperreactivity of host macrophages (13) or high reactivity of neutrophils to inflammatory stimuli (6) et al. When the disease progresses, all these pathways converge to induce uncontrolled inflammation characterized by overproduction of proinflammatory factors. These factors cause accelerated hematopoiesis, which leads to the generation of high numbers of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} immature myeloid cells and to the disappearance of mature Gr-1\textsuperscript{hi} neutrophils. Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells and lack of neutrophils may further interfere with the host immune response and exacerbate the infection. This concept implies that mechanisms driving TB disease at the initial stages of infection are diverse in different individuals. In contrast, mechanisms driving TB progression at the late stage of infection are common and include exacerbated inflammation, the generation of immature myeloid cells, and their accumulation in the periphery. If this concept is confirmed in humans, it may have an important practical outcome: it indicates that there is no need to search for exact cause that has driven severe disease in each individual; it might be possible to slow down disease progression by targeting MDSC cells and/or inflammatory reactions. In this respect, cotreatment of host with anti- *M. tuberculosis* and anti-inflammatory drugs opens new perspectives for efficient TB treatment (46).

In conclusion, to our knowledge, our study for the first time demonstrates that Gr-1–expressing cells abundantly accumulating at prelethal stage of TB do not belong to mature granulocytes, but rather represent immature myeloid cells able to suppress T cell responses. The accumulation of Gr-1\textsuperscript{dim}CD11b\textsuperscript{+} cells represents a hallmark of fatal TB infection in mice and may contribute to TB pathogenesis. These results change our view of mechanisms driving TB progression and suggest new potential cellular targets for TB immunotherapy.

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**References**


