Accumulation of Myeloid-Derived Suppressor Cells in the Lungs during Pneumocystis Pneumonia

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Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of hematopoietic precursors with the ability to adversely affect host immunity. They have been shown to accumulate in pathological conditions, such as cancer and some microbial diseases. In the mouse and rat models of Pneumocystis pneumonia (PCP), we found a distinct population of cells with MDSC-like morphology in the bronchoalveolar lavage (BAL) fluid, constituting up to 50% of the total cells in BAL fluid. These cells were not seen in the BAL fluid from normal animals or from Pneumocystis-infected animals that had been successfully treated for PCP with a combination of trimethoprim and sulfamethoxazole. With flow cytometry, these cells were found to express the characteristic MDSC surface markers Gr-1 and CD11b in mice or CD11b and His48 in rats. Using reverse transcription-PCR, we demonstrated that these cells produced high levels of arginase-1 and inducible nitric oxide synthase (iNOS) mRNA. These cells were shown to suppress CD4+ T-cell proliferation in response to stimulation by anti-CD3 and anti-CD28 antibodies. Adoptive transfer of these cells to normal mice caused lung damage, as indicated by elevated levels of albumin and lactate dehydrogenase in the BAL fluid. These experiments provide evidence of the presence of MDSCs in the lungs during PCP. Further studies on the roles of MDSCs in PCP are warranted in order to develop treatment strategies which can reduce the number of MDSCs and the damage caused by these cells.

Pneumocystis pneumonia (PCP) is one of the most common opportunistic diseases in immunocompromised patients, such as those with AIDS. However, many basic aspects of the pathogenesis of PCP and the host immune response to Pneumocystis infection remain unknown. Pneumocystis infections usually result in severe inflammatory damage to the lungs. Adjunctive corticosteroid therapy has been shown to alleviate the inflammation and improve the survival of AIDS patients with PCP (3, 7). Studies of various inflammatory components during Pneumocystis infection have found that CD8+ T cells fail to control organism burden but can accelerate lung injuries (8). In human patients, the severity of PCP is correlated with the relative number of neutrophils in bronchoalveolar lavage fluid (BALF) (1, 16); however, neutrophils have been shown to play no significant role in the pathogenesis of PCP in animals (28).

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid progenitor cells and immature myeloid cells. MDSCs have morphology similar to that of granulocytes or monocytes. In healthy individuals, immature myeloid cells are generated in the bone marrow and quickly differentiate into mature granulocytes, macrophages, or dendritic cells. Immature myeloid cells are generally absent from peripheral lymphoid organs. In mice, MDSCs are characterized by coexpression of the myeloid cell lineage differentiation antigen Gr-1 and CD11b. In rats, MDSCs are characterized by coexpression of His48 and CD11b (11). Expansion of MDSCs in the spleen and other peripheral organs has been found in cancers and some infectious diseases, such as toxoplasmosis (29), leishmaniasis (23), candidiasis (17), and helminthiasis (9, 26). The role of MDSCs in PCP has not been investigated.

In this study, we identified MDSCs as an important inflammatory component in the lungs of Pneumocystis-infected animals. Three weeks after initiation of Pneumocystis infection, MDSCs started to appear in the BALF, and their numbers continued to increase during the course of infection. The MDSCs from mice with PCP showed a CD11b+/Gr-1+ immunophenotype, expressed high levels of arginase-1 and inducible nitric oxide synthase (iNOS), and had the ability to suppress CD4+ T-cell proliferation.

MATERIALS AND METHODS

Rodent models of PCP. C57BL/6 mice and Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). All animals used in this study were female, with body weights of 18 to 20 g for mice and 120 to 140 g for rats. Animal studies were approved by the Indiana University Animal Care and Use Committee and carried out under the supervision of veterinarians. Immunosuppression of mice was achieved by intraperitoneal injection of 0.3 mg anti-CD4 MAb (clone GK1.5; Harlan, Indianapolis, IN) once a week until the mice were sacrificed. Three days after the initial injection, mice were transtracheally instilled with 2 × 10^6 Pneumocystis organisms in 50 μl sterile phosphate-buffered saline (PBS). Rats were immunosuppressed with 1.8 mg/ml dexamethasone in drinking water. One week after initiation of immunosuppression, rats were transtracheally instilled with 2 × 10^6 Pneumocystis organisms in 200 μl sterile PBS. The Pneumocystis organisms used as the inoculum were obtained from heavily infected rodent lungs and isolated as previously described (32). Tetracycline (0.73 g/liter) was added to the drinking water to prevent bacterial infections. Immunosuppressed, uninfected animals were used as controls. For the treatment group, Septa (Hi-Tech Pharmacal, Amityville, NY), which is the combination of trimethoprim (50 mg/kg of body...
weight/day) and sulfamethoxazole (250 mg/kg/day), was given orally once a day starting from 3 weeks postinoculation for mice and 2 weeks postinoculation for rats.

**Analysis of infection in animals transtracheally inoculated with Pneumocystis organisms.** At various time points after inoculation, animals were anesthetized by intramuscular injection of ketamine cocktail (ketamine hydrochloride, 80 mg/ml; acepromazine, 1.76 mg/ml; and atropine, 0.38 μg/ml) and then sacrificed by cardiac exsanguination. The severity of Pneumocystis infection was determined by scoring the number of organisms on histochemically stained impression smears of lung tissue as previously described (14). The smears were stained with Wright’s Giemsa stain for both trophozoite and cyst forms and with Grocott methenamine-silver nitrate stain for cyst forms. An infection score for each smear was determined by using a scale of 0 to 5 pluses, representing the following: +++++, more than 100 organisms per 1,000× microscopic field; ++++, between 11 and 100 organisms per 1,000× microscopic field; +++, between 1 and 10 organisms per 1,000× microscopic field; ++, between 2 and 9 in 50,000× microscopic fields; +, between 1 and 2 in 50,000× microscopic fields; and no pluses (or −), no organisms in 50,000× microscopic fields.

**Isolation of total BAL cells and Gr-1+ cells.** After animals were anesthetized as described above, lungs were lavaged with sterile saline (5 ml for rats and 1 ml for mice) at a ratio of 5:1 in a 96-well plate coated with anti-CD3 and anti-CD28 antibodies (eBioscience, San Diego, CA) at 37°C with 5% CO2. After 72 to 96 h, the cells were stained with fluorescence-labeled antibodies on ice for 1 h. Separate sets of cells were stained with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-labeled IgG isotype control antibody. After washing twice with 4 ml PBS, the stained cells were examined with a BD FACScan flow cytometer (BD Biosciences, San Jose, CA), and the flow cytometry data thus generated were analyzed with FlowJo software (Tree Star, Ashland, OR).

**T-cell proliferation assay.** A total of 5 × 10⁵ CD4+ T cells were isolated from spleens of normal mice by magnetic separation as described previously (31) and then labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA) at room temperature for 5 min. Cells were washed with PBS and incubated in RPMI 1640 medium at 37°C for 30 min. The CFSE-labeled T cells were incubated with Gr-1+ cells isolated from BALF of mice with PcP or from bone marrow of normal mice at a ratio of 5:1 in a 96-well plate coated with anti-CD3 and anti-CD28 antibodies (eBioscience, San Diego, CA) at 37°C with 5% CO₂. After 72 to 96 h, the cells were stained with PE-conjugated anti-CD4 antibody so that CD4+ T cells can be specifically gated and examined, and the CFSE fluorescence intensity of the PE-stained CD4+ T cells was determined by flow cytometry.

**Determination of albumin levels and LDH activity of BALF.** The first 1 ml of BALF was used for determination of albumin levels by using the QuantiChrom BCG albumin assay kit (BioAssay Systems, Hayward, CA) and lactate dehydrogenase (LDH) activity by using the LDH cytotoxicity assay kit (Cayman Chemical, Ann Arbor, MI) by following the manufacturers’ instructions.

**Morphological analysis of BAL cells.** One hundred microliters of a cell suspension was loaded into a cytospin chamber and spun for 5 min at 500 rpm (Cytospin 2; Shandon, Cheshire, England). Slides were air dried at room temperature for 5 min and stained with the LeukoStat staining kit (Fisher Scientific, Pittsburgh, PA).

**Flow cytometry analysis.** BAL cells were obtained from uninfected or Pneumocystis-infected animals. After blocking in 5% bovine serum albumin for 1 h, the cells were stained with fluorescence-labeled antibodies on ice for 1 h. Separate sets of cells were stained with phycoerythrin (PE) or fluorescein isothiocyanate (FITC)-labeled IgG isotype control antibody. After washing twice with 4 ml PBS, the stained cells were examined with a BD FACScan flow cytometer (BD Biosciences, San Jose, CA), and the flow cytometry data thus generated were analyzed with FlowJo software (Tree Star, Ashland, OR).

**Table 1. Pneumocystis organism burden in mice**

<table>
<thead>
<tr>
<th>Wk postinoculation</th>
<th>Uninfected</th>
<th>PcP</th>
<th>PcP with Septra treatment</th>
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<tbody>
<tr>
<td>3</td>
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<td>+</td>
<td>+</td>
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<td>5</td>
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<tr>
<td>7</td>
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*Scores are representative of 3 to 6 mice per group. Septra treatment was initiated 3 weeks after Pneumocystis inoculation.*
Real-time PCR of arginase-1 and iNOS. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration and purity were determined by spectrophotometry. cDNA was synthesized from total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and random primers; 0.2 μg of total RNA was used for each reaction with a total reaction volume of 20 μl. The reaction mixtures were incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. Two μl of each cDNA product was used for quantitative PCR analysis. Real-time reverse transcription-PCR (RT-PCR) for arginase-1 and iNOS were performed on a Smartcycler (Cepheid, Sunnyvale, CA) with SYBR master mix (Applied Biosystems) and the following primers: iNOS-F (5′-TGAACTTGAGCGAGCA-3′) and iNOS-R (5′-TTCATGTGATACGTTTCTGGCTCT-3′), as well as Arg-1-F (5′-CTCGAAGGAACTGAAAGGAAAG-3′) and Arg-1-R (5′-TTGGCAGATATGCAGGGAGT-3′). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were assayed in an identical manner as an internal control using primers GAPDH-F (5′-TGTGATGGACACCCCAAGCTCT-3′) and GAPDH-R (5′-CTGCGTTCCACCACTGCTG-3′). Data from each sample were normalized to the level of GAPDH gene expression and are shown as fold increase or percent relative to control cells.

Statistics. Data are presented as means ± standard deviations (SD) from the indicated number of experiments. Differences between groups were determined using the two-tailed Student’s t test and were considered statistically significant if P < 0.05.

RESULTS

BAL cells from mice with PcP showed morphological features characteristic of MDSCs. To study the cellular components in the alveolar space during Pneumocystis infection, we performed bronchoalveolar lavage on the immunosuppressed, Pneumocystis-infected C57BL/6 mice. BAL cells thus obtained were cytopun onto slides, stained with Giemsa, and examined under a light microscope. BAL cells from immunosuppressed-uninfected mice were processed in an identical manner to serve as controls. A very striking feature observed was the presence of cells with polymorphous nuclei, in addition to the normal-appearing alveolar macrophages in the BALF from Pneumocystis-infected mice (Fig. 1). These cells were heterogeneous in appearance and were generally smaller in size, with a higher nucleus-to-cytoplasm ratio compared to normal alveolar macrophages. Furthermore, these cells showed features of immature neutrophils or myeloid precursors. These immature-appearing cells had band- or ring-shaped nuclei and were hypogranular in the cytoplasm. Since these morphological features are consistent with those of myeloid-derived suppressed cells, we refer to these cells as MDSC-like cells. These cells started to appear in small numbers at 3 weeks postinoculation and became a significant proportion of total BAL cells 5 weeks after Pneumocystis inoculation. The number of MDSC-like cells in the
lung gradually increased over time as *Pneumocystis* infection became more severe (Table 1 and Fig. 1). No MDSC-like cells were observed in the BALF from uninfected mice or PcP mice treated with Septra for 2 or more weeks (Fig. 1).

**Gr-1⁺/CD11b⁺ cells accumulated in the lungs of mice with PcP and disappeared after eradication of *Pneumocystis* infection.** To determine whether these MDSC-like cells in the BALF of PcP mice display characteristic MDSC immunophenotypes, flow cytometry studies using Gr-1 and CD11b antibodies were performed on the BAL cells from control and *Pneumocystis*-infected mice at 5, 6, and 7 weeks after *Pneumocystis* inoculation. As seen in Fig. 2A, a population of Gr-1⁺/CD11b⁺ BAL cells was seen in samples from *Pneumocystis*-infected but not from uninfected mice at all 3 time points. The percentage of Gr-1⁺/CD11b⁺ cells in BALF increased gradually over the course of infection, from 26.2% ± 3.50% at 5 weeks and 41.0% ± 1.78% at 6 weeks to 50.7% ± 4.25% at 7 weeks postinoculation (Fig. 2B). Few Gr-1⁺/CD11b⁺ cells were observed in the BALF from immunosuppressed-uninfected animals or mice with PcP that had been treated with Septra for 2 or more weeks (Fig. 2A and B).

**Gr-1⁺/CD11b⁺ cells isolated from the BALF of PcP mice showed MDSC morphology.** To determine whether these Gr-1⁺/CD11b⁺ cells truly represent the MDSC-like cells observed in the BALF of animals with PcP by microscopy, this population of cells
was isolated using anti-Gr-1 antibody-conjugated Miltenyi magnetic beads. The isolated cells were analyzed for CD11b and Gr-1 by flow cytometry and were shown to have a purity greater than 90% (Fig. 3A). The purified cells were then cytopun on a slide, stained, and examined microscopically. As shown in Fig. 3B, these cells displayed exactly the same morphology as that of the MDSC-like cells shown in Fig. 1.

**CD11bc\(^+/\)His48\(^−\)** MDSCs accumulated in the lungs of rats with PcP and disappeared after eradication of *Pneumocystis* infection. To determine whether MDSCs are also present in rats with PcP, Sprague-Dawley rats infected with *Pneumocystis* for 7 weeks were lavaged and the BAL cells were examined microscopically. Similar to those from PcP mice, BAL cells from rats with PcP also contained a population of MDSC-like cells, which were absent from uninfected rats or PcP rats with 5 weeks of Septra treatment (Fig. 4A). Since rat MDSCs express His48 and CD11bc (11), flow cytometry studies using CD11bc and His48 antibodies were performed on the BAL cells from control (immunosuppressed-uninfected) and *Pneumocystis*-infected rats at 7 weeks after *Pneumocystis* inoculation. As seen in Fig. 4B, His48\(^+/\)CD11bc\(^+\) BAL cells were present in the samples from *Pneumocystis*-infected rats but not in those from uninfected rats or PcP rats treated with Septra for 5 weeks.

**Gr-1\(^+/\)CD11b\(^−\)** cells in BALF from PcP mice suppressed CD4\(^+\) T-cell proliferation. A major characteristic of MDSCs is the ability to suppress T-cell proliferation (27). To determine if the Gr-1\(^+/\)CD11b\(^−\) cells in the BALF from PcP mice at 6 weeks postinoculation are immunosuppressive, these cells were isolated and cocultured with CFSE-labeled CD4\(^+\) T cells isolated from the spleens of normal mice in the presence of anti-CD3 and anti-CD28 antibodies. Gr-1\(^+/\) cells from bone marrow of normal mice were used as controls. CFSE covalently binds to long-lived intracellular molecules. When a CFSE-labeled cell divides, its daughter cells inherit half the number of CFSE-tagged molecules; thus, each cell division can be measured by the corresponding decrease in cell fluorescence by flow cytometry. The percentage of cells with decreased fluorescence was calculated to determine the degree of proliferation. As expected, CD4\(^+\) T cells proliferated in response to stimulation by antibodies against CD3 and CD28, with 57.1% ± 9.8% of total cells showing decreased CFSE fluorescence (Fig. 5B). This proliferation was significantly suppressed by the MDSC-like cells from BALF of PcP mice, as evidenced by the observation that only 23.6% ± 8.2% of T cells showed decreased CFSE fluorescence (Fig. 5D). Gr-1\(^+\) cells from bone marrow of normal mice did not inhibit T-cell proliferation, as 61.9% ± 7.5% of the T cells had decreased CFSE fluorescence (Fig. 5C).
MDSCs from PcP mice expressed increased levels of arginase-1 and iNOS. Increased arginase-1 and iNOS expression have been described in MDSCs in murine models of cancer, and the role of both enzymes in the inhibition of T-cell functions has been well established (6). To determine whether the MDSCs accumulated in the lungs during PcP also express increased levels of these enzymes, total RNA was isolated from Gr-1$^{+}$ cells separated from the BALF of Pneumocystis-infected mice at 6 weeks postinoculation and reverse transcribed into cDNA. Gr-1$^{+}$ cells from bone marrow of normal mice were used as a control. The mRNA expression levels of arginase-1 and iNOS genes were determined by real-time PCR. As shown in Fig. 6, the arginase-1 mRNA expression levels of MDSCs from BALF of PcP mice were dramatically increased, by 2,801 $\pm$ 706-fold, compared to those of Gr-1$^{+}$ cells from bone marrow of normal mice ($P < 0.01$). The arginase-1 expression levels of MDSCs from PcP mice were also significantly increased (220 $\pm$ 60-fold; $P < 0.05$).

Adoptive transfer of MDSCs from PcP mice caused lung damage in normal mice. To study whether the accumulated MDSCs cause lung damage, $3 \times 10^5$ MDSCs isolated from BALF of PcP mice by magnetic separation were transtracheally injected into normal mice. Another group of normal mice was transtracheally injected with the same number of Gr-1$^{+}$ bone marrow cells to serve as a control. Lungs of injected mice were lavaged 60 h after the adoptive transfer and examined for disruption of the epithelial barrier by assaying the levels of albumin as well as destruction of lung cells by determining the LDH activity in BALF. As shown in Fig. 7, albumin levels in BALF were significantly increased in mice with adoptive transfer of MDSCs compared to those in BALF of control mice with adoptive transfer of bone marrow Gr-1$^{+}$ cells (6.71 $\pm$ 0.98 versus 3.72 $\pm$ 0.84 mg/dl; $P < 0.05$). The LDH activity in BALF was also significantly increased after adoptive transfer of MDSCs (90.5 $\pm$ 15.32 versus 54.67 $\pm$ 2.36; $P < 0.05$).

**DISCUSSION**

MDSCs were first described more than 20 years ago in tumor-bearing mice and in patients with cancer. However, their suppressive functions on the immune system have been intensively studied only in recent years. Most of our understanding about MDSCs is derived from studies of cancer-associated MDSCs. Although they have been shown to accumulate in many pathological conditions and to negatively regulate immune responses, relatively few studies have been focused on the roles of MDSCs in infectious diseases. We found in this study, for the first time, that MDSCs accumulate in the lungs during *Pneumocystis* infection. In our mouse model of PcP, MDSCs started to appear in the lungs 3 weeks after *Pneumocystis* inoculation and continued to accumulate in the lungs as the organism burden increased and lung inflammation became more severe. Since MDSCs were found in the lungs of both rats and mice with PcP, their appearance was due to *Pneumocystis* infection and not immunosuppression, as mice and rats were immunosuppressed by different means, rats with dexamethasone and mice with anti-CD4 antibody. Furthermore, MDSCs were not found in uninfected-immunosuppressed animals, and treatment of PcP animals with Septra eliminated MDSCs in their lungs.

MDSCs from animals with PcP showed granulocytic morphol-
ogy resembling that of neutrophils (Fig. 1 and 3). Interestingly, a study carried out in 1989 on 60 patients with PcP showed that 14 of 15 fatalities or cases of respiratory failure occurred in patients with greater than 5% neutrophils in BALF. Only one patient with less than 5% neutrophils in BALF died. It was concluded that the presence of neutrophils in BALF indicates advanced stages of PcP with a high rate of mortality (16). Given the close resemblance of the morphology between MDSCs and neutrophils and the fact that they both increase in numbers as infection progresses, it is possible that the neutrophils observed in BALF of these PcP patients were actually MDSCs. However, this speculation remains to be investigated.

MDSCs can be distinguished from myeloid precursors or neutrophils by their ability to inhibit T cells. MDSCs express high levels of arginase-1 and iNOS, leading to enhanced L-arginine catabolism. Depletion of this amino acid from the microenvironment leads to inhibition of T-cell proliferation and functions (21, 22). The products of L-arginine catabolism, including nitric oxide (NO), urea, and L-ornithine, also have suppressive effects on the immune system. NO can suppress T-cell function by inactivating JAK3 and STAT5 (2), inhibiting major histocompatibility complex class II expression (10), and inducing T-cell apoptosis (19). L-Ornithine can further be catabolized to various types of polyamines, such as spermine and spermidine, which we showed in our previous study induces apoptosis of alveolar macrophages (15). In the current study, Gr-1<sup>−</sup>/CD11b<sup>+</sup> BAL cells from PcP mice expressed high levels of arginase-1 and iNOS (Fig. 6) and were able to inhibit CD4<sup>+</sup> T-cell proliferation (Fig. 5). Collectively, these observations confirm the presence of MDSCs in the lung during PcP. Bone marrow Gr-1<sup>−</sup> cells were used as controls, since they share the same morphology and immunophenotypes as MDSCs (6). The control cells should have been isolated from the BAL of uninfected mice. Unfortunately, there are few MDSCs in the lungs of uninfected mice.

Activation of MDSCs in infectious diseases is mediated through the MyD88-dependent signaling pathways that are triggered by Toll-like receptors (TLRs) (5). TLR2, a MyD88-dependent signaling molecule, has been shown to mediate host immune response against Pneumocystis carinii pneumonia (13) and to activate NK cells to produce large amounts of gamma interferon in the presence of interleukin-12 (18). The roles of MDSCs in PcP are unknown. Our results showed that MDSCs caused direct damage to the lungs (Fig. 7); impaired clearance of organisms during PcP may be a result of immunosuppression caused by MDSCs. However, suppression of host immune response by MDSCs may also be beneficial to the host by diminishing the inflammatory damage. Due to the complex nature of the roles of MDSC in the pathogenesis of PcP, simple depletion of MDSC may not necessarily benefit the host. This is evidenced by the study of Swain et al. (25), in which treatment by anti-Gr-1 monoclonal antibody RB6 did not improve lung damage in a mouse model of PcP. It is our belief that induction of differentiation rather than simple elimination of MDSCs is the ideal way to treat the hosts during Pneumocystis infection.

Because MDSCs are immunosuppressive, some studies have been done to identify methods to eliminate them to increase host immune response to cancer, and administration of 2′, 2′-difluoro 2′-deoxycytidine (gemcitabine) to kill MDSCs in tumor-bearing mice has been shown to improve responses to immunotherapy (12, 24). Our results showing the existence of MDSCs in Pneumocystis-infected lungs and their ability to cause lung damage suggest that MDSCs are a target for treatment of or prophylaxis against PcP. To explore this possibility, we are currently investigating whether MDSCs are also present in the lungs of humans with PcP. Additional studies are needed to understand the roles of MDSCs in the pathogenesis of PcP and to enable the development of methods to target MDSCs as a therapy for PcP.

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REFERENCES


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