Paradoxical role of alveolar macrophage-derived granulocyte-macrophage colony-stimulating factor in pulmonary host defense post-bone marrow transplantation

Megan N. Ballinger,¹ Leah L. N. Hubbard,² Tracy R. McMillan,¹ Galen B. Toews,¹³ Marc Peters-Golden,¹ Robert Paine III,¹,³ and Bethany B. Moore¹

¹The Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, ²The Graduate Program in Immunology, and ³The Department of Veterans Affairs Medical Center, University of Michigan, Ann Arbor, Michigan

Submitted 2 August 2007; accepted in final form 26 April 2008

Ballinger MN, Hubbard LL, McMillan TR, Toews GB, Peters-Golden M, Paine R 3rd, Moore BB. Paradoxical role of alveolar macrophage-derived granulocyte-macrophage colony-stimulating factor in pulmonary host defense post-bone marrow transplantation. Am J Physiol Lung Cell Mol Physiol 295: L114–L122, 2008. First published May 2, 2008; doi:10.1152/ajplung.00309.2007.—Impaired host defense post-bone marrow transplant (BMT) and have shown that these mice are more susceptible to a pulmonary Pseudomonas aeruginosa infection than are untransplanted control mice. The dysfunction of both AMs and PMNs in these mice was associated with and largely explained by overproduction of prostaglandin E₂ (PGE₂) post-BMT (5). The mechanism(s) responsible for the elevated production of PGE₂ post-BMT have not been elucidated. Additionally, AMs from BMT mice are phenotypically immature (38), and it is likely that other as yet unidentified transplant-related alterations influence the behavior of innate immune cells.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multifunctional cytokine that promotes the differentiation and survival of myeloid precursors (21). Previous work has shown that GM-CSF has a role in stimulating the terminal differentiation of AMs through the transcription factor PU.1 (9, 47). GM-CSF also upregulates the activity of cytosolic phospholipase A₂ (cPLA₂) (11), and PU.1 can increase cyclooxygenase (COX)-2 expression. Both of these enzymes, cPLA₂ and COX-2, are necessary for PGE₂ synthesis, which has been shown to be upregulated both in HSCT patients (12) and in BMT mice (5). Limiting the production of PGE₂ post-BMT, by pharmacological or genetic strategies, improves host defense against P. aeruginosa (5). GM-CSF is also known to modulate host defense against this pathogen. Complete GM-CSF−/−

Hematopoietic stem cell transplantation (HSCT) is a therapeutic option for a variety of malignant diseases and inherited disorders including hematologic cancers, immune deficiencies, and as a rescue therapy following irradiation of solid tumors (18). Although beneficial in many cases, the success of HSCT is limited due to many serious infectious (31) and noninfectious (10, 30, 43) complications. Over half of all HSCT patients suffer from pulmonary complications, and nearly one-third of these patients require admission to the intensive care unit (6, 31). Pneumonia remains the leading infectious cause of death following transplantation despite the implementation of numerous prophylactic strategies and advances in diagnosis and treatment. The lung has the largest interface with the outside environment of any internal organ in the body. To maintain health, the body uses cellular mediators, such as cytokines, chemokines, and eicosanoids, to aid in the recruitment of cells and the containment and clearance of microorganisms (48). HSCT patients manifest increased susceptibility to infections for extended periods (months to years) following transplantation extending long after engraftment (3, 6), which may be explained by the fact that both alveolar macrophages (AMs) and polymorphonuclear leukocytes (PMNs) from these patients are defective in their ability to phagocytose and kill microorganisms (51, 55).

We have previously established a murine model of gram-negative bacterial infection following syngeneic bone marrow transplant (BMT) and have shown that these mice are more susceptible to a pulmonary Pseudomonas aeruginosa infection than are untransplanted control mice. The dysfunction of both AMs and PMNs in these mice was associated with and largely explained by overproduction of prostaglandin E₂ (PGE₂) post-BMT (5). The mechanism(s) responsible for the elevated production of PGE₂ post-BMT have not been elucidated. Additionally, AMs from BMT mice are phenotypically immature (38), and it is likely that other as yet unidentified transplant-related alterations influence the behavior of innate immune cells.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multifunctional cytokine that promotes the differentiation and survival of myeloid precursors (21). Previous work has shown that GM-CSF has a role in stimulating the terminal differentiation of AMs through the transcription factor PU.1 (9, 47). GM-CSF also upregulates the activity of cytosolic phospholipase A₂ (cPLA₂) (11), and PU.1 can increase cyclooxygenase (COX)-2 expression. Both of these enzymes, cPLA₂ and COX-2, are necessary for PGE₂ synthesis, which has been shown to be upregulated both in HSCT patients (12) and in BMT mice (5). Limiting the production of PGE₂ post-BMT, by pharmacological or genetic strategies, improves host defense against P. aeruginosa (5). GM-CSF is also known to modulate host defense against this pathogen. Complete GM-CSF−/−
mice are more susceptible to *P. aeruginosa* (7). In these mice, production of GM-CSF by lung parenchyma only [under control of the surfactant protein C (SP-C) promoter] restores host defense against *P. aeruginosa* (7). Thus we sought in this study to evaluate the production and compartmentalization of GM-CSF post-BMT and to determine whether this cytokine plays a role in the regulation of AM function and/or eicosanoid generation post-BMT.

**MATERIALS AND METHODS**

*Animals.* Wild-type (WT) C57BL/6 (Ly5.1; CD45.2) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6Ly5.2 (CD45.1) mice were purchased from the National Cancer Institute Frederick Cancer Research Facility (Frederick, MD). Transplantation between CD45.1 and CD45.2 mice allowed donor vs. host leukocytes to be distinguished by staining for the CD45.1 and CD45.2 alleles. GM-CSF−/− mice were generated by Dranoff et al. (19) and backcrossed 8 generations against C57BL/6 CD45.2 mice. All of the GM-CSF−/− mice were used by 6 mo of age, before developing noticeable pathology associated with pulmonary alveolar proteinosis (19).

Mice were housed under specific pathogen-free conditions and monitored daily by veterinary staff. All mice were euthanized by CO2 asphyxiation. The University of Michigan Committee on Use and Care of Animals approved these experiments.

*Bone marrow transplantation.* Recipient mice received 13 Gy of total body irradiation (137Cs source) delivered in two fractions separated by 3 h. A cell mixture of 5 × 10⁶ bone marrow (BM) cells was resuspended in serum-free media (SFM; DMEM, 0.1% BSA, 1% penicillin-streptomycin, 1% glutamine, 0.1% amphotericin B) and these were transplanted into syngeneic recipients via tail vein infusion (0.2 ml total volume). All experiments with BMT mice were performed 5–8 wk post-BMT. AMs are 78 ± 5.7% donor-derived at this time. PMNs are >98% donor-derived at this time point.

*Intratracheal infection with* *P. aeruginosa.* A 1:1,000 dilution of *P. aeruginosa* PAO1 stock was grown in Tryptic Soy Broth (Difco, Detroit, MI) by shaking for 18 h at 37°C. Bacterial concentration was determined by measuring the amount of absorbance at 600 nm compared with a predetermined standard curve. Bacteria were then diluted to the desired concentration for inoculation, and animals were anesthetized and given the intratracheal (i.t.) inoculum of *P. aeruginosa* or saline as previously described (5).

*Quantification of bacterial burden in lung and blood.* Following an i.t. inoculum with *P. aeruginosa* cells, mice were euthanized at 24 h. Blood and lung were collected and processed as previously described (5). Using serial 10-fold dilutions, 10 μl of each specimen (lung and blood) was plated on blood agar plates and incubated at 37°C. Bacterial colonies were counted at 24 h post-i.t. as colony-forming units (CFU) per milliliter of blood or CFU per whole lung.

*Harvesting resident AMs and recruited lung PMNs.* Resident AMs from mice were obtained via ex vivo lung lavage as previously described (5). The cells were enumerated by counting on a hemocytometer before plating. Cells were allowed to adhere to tissue culture plates for 1 h in SFM and were then washed to remove nonadherent cells, resulting in >95% pure AM culture as determined by modified Wright–Giemsa stain.

Lung PMNs were obtained by lung lavage 18 h post-i.t. injection with 25 μg of LPS derived from *P. aeruginosa* (Sigma, St. Louis, MO) (5). At this time point, the percentage of PMNs in the lavage ranged from 87 to 93% as determined by differential staining. PMNs were collected by centrifugation, washed twice, allowed to adhere for 30 min in SFM, and then immediately used for analysis.

*In vitro phagocytosis assay.* AMs were harvested, and the ability of these cells to phagocytose unopsonized bacteria was examined using fluorescently labeled *Escherichia coli* bioparticles (Vybrant phagocytosis assay; Molecular Probes, Eugene, OR) according to manufacturer’s instructions as previously described (5).

**Tetrazolium dye reduction assay of bacterial killing.** The ability of AMs and PMNs to kill *P. aeruginosa* was quantified using a tetrazolium dye reduction assay (46). Briefly, AMs and PMNs were collected. PMNs were cultured for 30 min in SFM and then assayed immediately, whereas AMs were cultured overnight in complete media (DMEM, 10% FBS, 1% penicillin-streptomycin, 1% glutamine, 0.1% amphotericin B) before assay. The survival of intracellular bacteria was determined by analysis of the amount of bacteria in a control plate that was only allowed to phagocytose the bacteria but not kill it compared with the amount of bacteria on the experimental plate, in which the cells were given time to kill the ingested bacteria. Results were expressed as percentage of survival of ingested bacteria, where the survival of ingested bacteria = (A595 control plate/A595 experimental plate) × 100%.

**Real-time RT-PCR.** Real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA). Gene-specific primers and probes were designed using Primer Express software (Applied Biosystems) and were previously published (5). AMs or BM-derived macrophages (BMDMs) were harvested from each group, and the mRNA was extracted using TRIzol reagent (Invitrogen). For each experiment, samples from mice (n = 2–3) were run in triplicate. The average cycle threshold (Ct) was determined for each sample from a given experiment. Relative gene expression (using the formula 2−ΔΔCt) was calculated using the comparative Ct method (20), which assesses the difference in gene expression between the gene of interest (EP2) and an internal standard gene (β-actin) for each sample to generate the ΔΔCt. The average of the control sample was set to 1 for each experiment, and the relative gene expression for each experimental sample was compared with that.

**Enzyme immunoassay.** The production of TNF-α and GM-CSF was measured using Opti-EIA kits (BD Pharmingen, San Diego, CA). The levels of eicosanoids [PGE2, total cysteinyl leukotrienes (cys-LTs: LTC4, LTD4, and LTE4)] were measured using specific enzyme immunoassays (EIA) (Cayman Chemical, Ann Arbor, MI) in the presence or absence of calcium ionophore (A23187) according to the manufacturer’s instructions.

**cAMP measurements.** AMs were harvested by lung lavage, plated at 0.5 × 10⁶ cells per well in a 12-well plate, and adherence purified in SFM for 1 h. Cells were then incubated in complete media overnight. The next day, the media was removed and replaced either with SFM or SFM containing 1 μM PGE2 for 15 min at 37°C. Medium was removed, and 0.25 ml of 0.1 M HCl was added to the cells for 20 min at room temperature to prepare cell lysates. This cell lysate was then analyzed for cAMP levels using the Assay Designs (Ann Arbor, MI) CAMP EIA kit according to manufacturer’s instructions.

**Statistical analysis.** Statistical significance was analyzed using Prism 3.0 statistical program (GraphPad Software, San Diego, CA). Comparisons between two experimental groups were performed with Student’s t-test. Comparisons among three or more experimental groups were performed with ANOVA with a post hoc Bonferroni test to determine significance. A value of P < 0.05 was considered significant.

**RESULTS**

**AMs from BMT mice overproduce GM-CSF.** GM-CSF is known to regulate AM maturation (39) and the production of eicosanoids by AMs (11); therefore, we investigated the production of GM-CSF by AMs from untransplanted control and BMT (CD45.2 BM into CD45.1 recipient) mice. For comparison, we analyzed AMs from CD45.1 mice that had been transplanted with BM from GM-CSF−/− mice (GM-CSF−/− BMT). AMs were cultured overnight in the presence
or absence of LPS, and cell supernatants were collected 24 h later and analyzed for GM-CSF production by specific ELISA. Figure 1 demonstrates that AMs from control BMT mice produce significantly elevated levels of GM-CSF both at baseline (Fig. 1A) and when stimulated with LPS (Fig. 1B). As expected, levels of GM-CSF produced from AMs in GM-CSF−/− BMT mice were extremely low both with and without LPS stimulation.

**GM-CSF levels in lung homogenates are reduced in BMT mice postinfection.** We next assessed the production of GM-CSF by the lung parenchyma. Figure 2A demonstrates the levels of GM-CSF that were detectable in uninfected lung homogenates following the removal of AMs via lung lavage. The levels of GM-CSF that are detectable in the lung parenchyma before infection were significantly diminished in both the control BMT and the GM-CSF−/− BMT group. Next, untransplanted control, control BMT, and GM-CSF−/− BMT mice were infected with *P. aeruginosa* 6 wk posttransplant. Twenty-four hours later, lungs were collected and analyzed for expression of GM-CSF by specific ELISA (Fig. 2B). Overall, levels of GM-CSF measured in the parenchyma of infected mice were much greater than in uninfected mice. However, even postinfection, the levels of GM-CSF measured in the lungs of control BMT mice were significantly lower than levels in infected untransplanted control mice. Interestingly, in the absence of AM-derived GM-CSF post-BMT, the ability of the lung to produce GM-CSF in response to infection was improved.

**GM-CSF−/− BMT mice are protected from a gram-negative bacterial infection in vivo.** To determine whether the excess production of GM-CSF by AMs posttransplant contributed to the impaired pulmonary host defense of these mice, the response of control BMT, GM-CSF−/− BMT, and untransplanted control mice to bacterial infection was determined 24 h post-*P. aeruginosa* inoculation. As previously shown, the control BMT mice have an enhanced susceptibility to *P. aeruginosa* infections, as reflected by elevated CFU in the lung and blood (Fig. 3, A and B). In contrast, the GM-CSF−/− BMT mice had lower bacterial burdens in their lungs (Fig. 3A) and less dissemination into the blood (Fig. 3B).

**Absence of GM-CSF in hematopoietic cells restores the function of AMs and PMNs post-BMT.** Our previous studies (5, 38) indicated that AMs from BMT mice display defects in both phagocytosis and killing, whereas PMNs from BMT mice display only killing defects. Thus AMs and PMNs from untransplanted control, control BMT, and GM-CSF−/− BMT mice were collected, and their functional abilities were assessed. When the ability of hematopoietic cells to produce
GM-CSF post-BMT was eliminated, GM-CSF−/− BMT AMs were able to phagocytose bacteria significantly more efficiently than control BMT AMs (Fig. 4A). Similarly, GM-CSF−/− BMT AMs were able to kill ingested bacteria significantly better than both control BMT and untransplanted control mice (Fig. 4B). Figure 4C shows that the PMNs from GM-CSF−/− BMT mice tended to kill better than control BMT mice, but the improvement did not reach the level of PMNs from untransplanted control mice.

**AMs from GM-CSF−/− BMT mice produce elevated amounts of cys-LTs and PGE2.** We next wanted to determine whether the improvements in the function of innate immune cells post-GM-CSF−/− BMT could be attributed to normalization of eicosanoid levels. Thus supernatants were collected from untransplanted control, control BMT, and GM-CSF−/− BMT AMs cultured overnight, and the amount of eicosanoids produced were measured by specific EIA. We have previously published that BMT AMs produce elevated levels of PGE2 (5), and these findings are confirmed again in this study (Fig. 5A). Surprisingly, the GM-CSF−/− BMT AMs continue to overproduce PGE2 at levels that are not significantly different from control BMT mice. To determine whether the increase in PGE2 represented a generalized increase in all prostaglandins or a specific increase in PGE2, we measured the production of 6-keto-prostaglandin F1α (6-keto-PGF1α), a stable metabolite of prostacyclin, in the supernatants of the AMs from all three groups of mice (Fig. 5B). Overall, the levels of 6-keto-PGF1α were much lower than the levels of PGE2 produced by AMs. Although there was a trend toward elevated levels of this prostanoid post-BMT, in these experiments, this difference did not reach statistical significance. Cells were also stimulated for 1 h in the presence of the calcium ionophore A23187 to provide a maximal stimulus for cys-LT production. Although control BMT mice displayed defects in the production of cys-LTs as previously noted (5), there was no difference in the production of cys-LTs in GM-CSF−/− BMT AMs compared with untransplanted control AMs (Fig. 5C).

**EP2 receptor expression on GM-CSF−/− BMT AMs.** We have shown that the inhibitory actions of PGE2 on AM function are mediated via the EP2 receptor (2, 5). AMs from control BMT mice have previously been shown to express elevated levels of EP2 mRNA compared with AMs from untransplanted control mice (5). Thus we wanted to analyze the expression of EP2 in AMs from GM-CSF−/− BMT mice. As antibodies to EP2 are nonspecific in the mouse, we used real-time RT-PCR to analyze EP2 mRNA levels in AMs from untransplanted control, control BMT, and GM-CSF−/− BMT mice (Fig. 6A). As anticipated, AMs from control BMT mice have increased mRNA for EP2 (5). Interestingly, EP2 mRNA expression in GM-CSF−/− BMT AMs was suppressed compared with that from control BMT AMs and was not different than the expression in control AMs.

The responsiveness of AMs to PGE2 correlates with EP2 receptor levels. To determine whether the altered expression of EP2 in the various groups of mice had functional significance, we measured the production of cAMP in response to PGE2 stimulation in AMs isolated from untransplanted control, control BMT, and GM-CSF−/− BMT mice (Fig. 6B). Stimulation of untransplanted control AMs with 1 μM PGE2 for 15 min resulted in increased cAMP generation. The same stimulation of AMs from control BMT mice (which have higher levels of EP2) resulted in significantly higher levels of cAMP generation. In contrast, stimulation of AMs from GM-CSF−/− BMT mice (which had the lowest levels of EP2) resulted in the least accumulation of cAMP in response to PGE2 stimulation. These data suggest that the regulation of EP2 receptor expression dictates the responsiveness of the AMs to PGE2 stimulation.

**TNF-α production is restored in GM-CSF−/− BMT mice postinfection.** Impaired host defense against *P. aeruginosa* post-BMT has been linked with diminished production of...
normal host defense. Topoietic cells contributes to impaired host defense, whereas BMT, and WT into GM genetic ablation of GM-CSF in the parenchymal cells (WT into GM-P. aeruginosa once again that control BMT mice are more susceptible to with P. aeruginosa and bacterial killing was assayed as above, Activity of control AMs was set to 100%. Defective killing is indicated by an increase in the amount of surviving bacteria, n = 5. C: PMNs were collected from each group of mice, and bacterial killing was assayed as above, n = 5.

Fig. 4. AM and polymorphonuclear leukocyte (PMN) function is improved in GM-CSF−/− BMT mice. A: AMs were harvested from untransplanted control, control BMT, or GM-CSF−/− BMT mice and analyzed for the ability to ingest FITC-labeled bacteria. The phagocytic ability of the control AMs was set to 100%. Defective killing is indicated by an increase in the amount of surviving bacteria, n = 5. B: the ability of the AMs from each group of mice to kill ingested bacteria was measured using a tetrazolium dye reduction assay. Activity of control AMs was set to 100%. Defective killing is indicated by an increase in the amount of surviving bacteria, n = 5.

Our studies have demonstrated a curious dichotomy in the homeostatic regulation of GM-CSF post-BMT. Although AMs overproduce GM-CSF post-BMT, parenchymal production of this cytokine is impaired both at baseline and in the setting of infection in control BMT mice. We next analyzed the contribution of AM-derived GM-CSF post-BMT by creating GM-CSF−/− BMT chimeric mice. The GM-CSF−/− BMT mice are protected from an in vivo P. aeruginosa infection as shown by decreased bacterial burden in the lung and decreased dissemination into the blood compared with control BMT mice. The defective phagocytosis and killing phenotype of the control BMT AMs and the defective killing phenotype of the control BMT PMNs were also reversed in lung phagocytes from GM-CSF−/− BMT mice. Surprisingly, these alterations were seen despite the continued high level production of PGE2. To understand how the GM-CSF−/− BMT AMs could function appropriately in the face of elevated PGE2 levels, we also examined the expression of EP2 receptors and the production of cys-LTs by AMs from the GM-CSF−/− BMT mice. In control BMT mice, the elevated production of PGE2 is also associated with enhanced mRNA expression of EP2, the receptor that PGE2 signals through to inhibit host defense in AMs (2, 5, 45). The elevation in EP2 levels correlates with robust production of cAMP in AMs from control BMT mice when stimulated with PGE2. In contrast, the AMs from the GM-CSF−/− BMT mice display reduced PGE2 mRNA levels and reduced cAMP generation in response to PGE2 stimulation. Thus the inhibitory signaling by PGE2 in the GM-CSF−/− BMT cells is limited even though production of PGE2 is not. Additionally, the AMs from the GM-CSF−/− BMT mice retain their ability to produce cAMP, eicosanoids that promote phagocytosis and killing in AMs (5, 35). In vivo the GM-CSF−/− BMT mice also retain the ability to produce TNF-α in response to infection. Interestingly, parenchymal production of GM-CSF in the lung post-P. aeruginosa was equivalent to control in the GM-CSF−/− BMT mice. These results suggest that homeostatic production of GM-CSF in the lung is tightly regulated, potentially via hematopoietic/structural cell cross talk. In addition, our results suggest that AM-derived GM-CSF is detrimental to host defense post-

DISCUSSION

Our studies have demonstrated a curious dichotomy in the homeostatic regulation of GM-CSF post-BMT. Although AMs overproduce GM-CSF post-BMT, parenchymal production of this cytokine is impaired both at baseline and in the setting of infection in control BMT mice. We next analyzed the contribution of AM-derived GM-CSF post-BMT by creating GM-CSF−/− BMT chimeric mice. The GM-CSF−/− BMT mice are protected from an in vivo P. aeruginosa infection as shown by decreased bacterial burden in the lung and decreased dissemination into the blood compared with control BMT mice. The defective phagocytosis and killing phenotype of the control BMT AMs and the defective killing phenotype of the control BMT PMNs were also reversed in lung phagocytes from GM-CSF−/− BMT mice. Surprisingly, these alterations were seen despite the continued high level production of PGE2. To understand how the GM-CSF−/− BMT AMs could function appropriately in the face of elevated PGE2 levels, we also examined the expression of EP2 receptors and the production of cys-LTs by AMs from the GM-CSF−/− BMT mice. In control BMT mice, the elevated production of PGE2 is also associated with enhanced mRNA expression of EP2, the receptor that PGE2 signals through to inhibit host defense in AMs (2, 5, 45). The elevation in EP2 levels correlates with robust production of cAMP in AMs from control BMT mice when stimulated with PGE2. In contrast, the AMs from the GM-CSF−/− BMT mice display reduced PGE2 mRNA levels and reduced cAMP generation in response to PGE2 stimulation. Thus the inhibitory signaling by PGE2 in the GM-CSF−/− BMT cells is limited even though production of PGE2 is not. Additionally, the AMs from the GM-CSF−/− BMT mice retain their ability to produce cAMP, eicosanoids that promote phagocytosis and killing in AMs (5, 35). In vivo the GM-CSF−/− BMT mice also retain the ability to produce TNF-α in response to infection. Interestingly, parenchymal production of GM-CSF in the lung post-P. aeruginosa was equivalent to control in the GM-CSF−/− BMT mice. These results suggest that homeostatic production of GM-CSF in the lung is tightly regulated, potentially via hematopoietic/structural cell cross talk. In addition, our results suggest that AM-derived GM-CSF is detrimental to host defense post-

AJP-Lung Cell Mol Physiol • VOL 295 • JULY 2008 • www.ajplung.org
BMT, whereas parenchymal cell production of GM-CSF is necessary for host defense.

GM-CSF is a multifunctional cytokine known to stimulate the differentiation and function of myeloid cells (21, 24). In fact, GM-CSF has successfully been used in autologous and allogeneic HSCT patients to accelerate PMN and platelet engraftment and to limit mucositis (17, 23, 34). Although GM-CSF does accelerate myeloid engraftment posttransplant, there is little evidence that GM-CSF treatment actually influences infections (23, 25) and some evidence that GM-CSF treatment increases the patient’s risk for the development of anticoagulant deficiency (22). In animal models, it is clear that both over- and underproduction of GM-CSF can be associated with disease, but the results are often contradictory and model dependent. Transgenic overproduction of GM-CSF via a retroviral promoter results in the accumulation and activation of macrophages, vision impairment, and muscle wasting disease (32). Adenoviral delivery of GM-CSF to the lung results in lung eosinophilia, macrophage accumulation, and fibrosis (52–54); however, in mice that overexpress GM-CSF in epithelial cells via the SP-C promoter (SP-C-GM-CSF mice), epithelial cell hyperplasia was noted, but fibrosis was not (44). Additionally, GM-CSF−/− mice are more susceptible to fibrosis induced by bleomycin (37), whereas SP-C-GM-CSF mice are

BMT AMs do not overexpress the inhibitory EP2 receptor and have diminished cAMP responses to PGE2 stimulation. A: AMs were collected from untransplanted control, control BMT, or GM-CSF−/− BMT mice, and cultured for 1 h before cell supernatants were collected and analyzed for production of PGE2 (A), 6-keto-prostaglandin F1α, (6-keto-PGF1α; a stable metabolite of PGI2; B), or cys-LTs following a 1-h stimulation with 5 μM calcium ionophore (C) by specific EIA, n = 6.

GM-CSF REGULATES INNATE IMMUNITY POST-BMT

Fig. 5. GM-CSF−/− BMT AMs produce both cysteinyl leukotrienes (cys-LTs) and PGE2. AMs were collected from untransplanted control, control BMT, or GM-CSF−/− BMT mice and cultured for 24 h before cell supernatants were collected and analyzed for production of PGE2 (A), 6-keto-prostaglandin F1α, (6-keto-PGF1α; a stable metabolite of PGI2; B), or cys-LTs following a 1-h stimulation with 5 μM calcium ionophore (C) by specific EIA, n = 6.

Fig. 6. GM-CSF−/− BMT AMs do not overexpress the inhibitory EP2 receptor and have diminished cAMP responses to PGE2 stimulation. A: AMs were collected from untransplanted control, control BMT, or GM-CSF−/− BMT mice, and total RNA was prepared from each population. RNA was then analyzed by real-time RT-PCR for expression of EP2 and β-actin. Normalized values for EP2 expression in untransplanted control AMs were set to 1, n = 4. B: AMs were collected from untransplanted control, control BMT, or GM-CSF−/− BMT mice. Following overnight culture, cells were stimulated with serum-free media or with 1 μM PGE2 for 15 min at 37°C. Cell lysates were then prepared in 0.1 N HCl and analyzed for cAMP generation, n = 4.
protected against exogenous fibrotic insults (13). Genetic ablation of GM-CSF in all cells is associated with alveolar proteinosis caused by a defect in AM clearance of surfactant (reviewed in Ref. 49). Thus GM-CSF levels must be tightly regulated to maintain lung homeostasis.

The role of GM-CSF in infection is complex. GM-CSF−/− mice have impaired host defense in many models of both bacterial and fungal pneumonia (7, 14, 33, 40). Treatment with GM-CSF can reverse the susceptibility of mice to Klebsiella pneumoniae in the setting of hyperoxic lung injury as well (4). However, GM-CSF has also been shown to promote viral replication in other situations (8). Our results suggest that AM-specific overproduction of GM-CSF post-BMT and diminished production of GM-CSF in the lung parenchyma in combination is detrimental for host defense in the control BMT mice. Interestingly, in the GM-CSF−/− BMT mice, production of GM-CSF in AMs was eliminated, but parenchymal production of GM-CSF was improved postinfection. Previous studies have clearly demonstrated that AM-derived GM-CSF is not critical for host defense against P. aeruginosa provided that parenchymal production of GM-CSF is present (7). Our results mirror these findings and suggest that AM-derived GM-CSF is dispensable for host defense post-BMT but that parenchymal-derived GM-CSF is necessary as chimeric WT into GM-CSF−/− BMT mice were impaired in their ability to clear a P. aeruginosa infection. Both alveolar epithelial cells and fibroblasts are sources of parenchymal lung GM-CSF (13, 15). AMs from BMT mice express similar levels of both the α- and β-subunits of the GM-CSF receptor, and the proliferative response of these AMs to exogenous GM-CSF are normal (data not shown). Thus the defects in host defense postcontrol BMT do not appear to be related to a loss of AM responsiveness to GM-CSF.

GM-CSF is known to upregulate the release of arachidonic acid from membrane phospholipids in macrophages via induction of cPLA2 (11). Once liberated, PGE2 is produced from arachidonic acid via the actions of either COX-1 or COX-2 (41). GM-CSF is also able to upregulate the expression of PU.1 (47), a transcription factor that can enhance COX-2 expression (28). Thus it seemed likely that the overproduction of GM-CSF in AMs post-BMT would drive the production of PGE2 in these same cells. However, this is not what we observed. AMs from GM-CSF−/− BMT mice still produce elevated amounts of PGE2 post-BMT despite the absence of the GM-CSF gene in these cells. The increased expression of PGE2 in the GM-CSF−/− BMT mice cannot be explained by excess GM-CSF in the lung environment preinfection either. Thus it is likely that COX-2 induction occurs via other transplant-related mechanisms such as cytokine release or oxidative stress in these mice. Interestingly, the GM-CSF−/− mice did not show the upregulation of EP2 nor the enhanced generation of cAMP in response to EP2 signaling that is noted in control BMT mice. In fact, AMs from GM-CSF−/− BMT mice display reduced levels of EP2 and have diminished cAMP responses following EP2 stimulation. This likely explains why AMs from GM-CSF−/− BMT mice show preserved host defense function in the face of elevated PGE2. EP2 is known to be upregulated by LPS (27, 29) and downregulated by PGE2 binding in a negative feedback loop (26). GM-CSF has not been reported previously to regulate EP2 expression, and our results do not distinguish between whether this is a direct or indirect effect.

A second feature of the AMs from GM-CSF−/− BMT mice was the retention of cys-LT production in these cells. Unmanipulated GM-CSF−/− mice show defects in synthesis of leukotrienes (7), SP-C-driven expression of GM-CSF in GM-CSF−/− mice has been shown to partially restore cys-LT synthesis by AMs (39). AMs from control BMT mice are defective in production of cys-LTs, and exogenous addition of

---

**Fig. 7.** GM-CSF−/− BMT mice produce normal levels of TNF-α in response to infection. Untransplanted control, control BMT, or GM-CSF−/− BMT mice were infected with P. aeruginosa, and lung homogenates were harvested 24 h later for determination of TNF-α levels by specific EIA, n = 4.

**Fig. 8.** Parenchymal-derived GM-CSF is necessary for host defense post-BMT. Untransplanted control, control BMT, GM-CSF−/− BMT, or wild-type into GM-CSF−/− BMT mice were inoculated with 4.69 log10 P. aeruginosa intratracheally. Twenty-four hours later, lungs (A) and blood (B) were harvested and plated in serial dilutions for CFU analysis, n = 5.
leukotriene D₄ augments phagocytosis of AMs post-BMT(5). GM-CSF is known to restore cys-LT production in both monocytes and PMNs from HIV-infected individuals (16), and the effects of GM-CSF on arachidonic acid release (11) would be expected to promote cys-LT synthesis as well. Thus the restoration of cys-LT production in the GM-CSF/–/– BMT mice may be explained by the ability of parenchymal production of GM-CSF to be induced in the GM-CSF/–/– BMT mice in a manner similar to that found in the SP-C-GM-CSF transgenic mice (39). It is likely that the retention of cys-LT production in the GM-CSF/–/– BMT AMs contributes to the improved phagocytosis and killing noted (42). Taken together, these results suggest that homeostatic levels of GM-CSF are necessary for appropriate regulation of eicosanoid production and signaling.

Impaired pulmonary host defense post-BMT is known to be associated with inappropriate induction of TNF-α in response to infection (38). Following a P. aeruginosa infection, both lung homogenates and isolated AMs from GM-CSF/–/– BMT mice are able to produce equivalent amounts of TNF-α as samples from control untransplanted mice. Thus genetic ablation of GM-CSF production by hematopoietic cells post-BMT is associated with improved production of proinflammatory cytokines in response to infection. It is possible that TNF-α levels are maintained in this model because of reduced negative signaling via EP2 in AMs (1, 36, 50) due to enhanced cys-LT signaling in the GM-CSF/–/– BMT mice or due to elevated parenchymal production of GM-CSF compared with the control BMT mice postinfection. This would be analogous to previous studies that demonstrated that impaired TNF-α production in AMs from GM-CSF/–/– mice was restored if GM-CSF was provided transgenically by lung epithelial cells (39).

In summary, our results indicate that control BMT mice are characterized both by overproduction of GM-CSF in AMs and underproduction of GM-CSF in the lung parenchyma post-BMT and that together these alterations result in impaired innate immunity. Genetic ablation of GM-CSF in the hematopoietic, but not structural, cells during BMT is associated with improved clearance of bacteria from both lung and blood, improved AM phagocytosis and killing, and normalized expression of cys-LTs and TNF-α. AMs from GM-CSF/–/– BMT mice also have reduced levels of EP2 and diminished inhibitory cAMP generation on stimulation with PGE2. The GM-CSF/–/– BMT mice also retain GM-CSF production in the lung environment postinfection. In contrast, genetic ablation of GM-CSF in the structural cells post-BMT results in impaired host defense. These results suggest that cross talk between hematopoietic cells and lung parenchymal cells is likely important in regulating the homeostatic levels of GM-CSF produced. These studies further suggest that the overproduction of AM-derived GM-CSF post-BMT is detrimental to host defense, whereas parenchymal-production of GM-CSF post-BMT is necessary for effective innate immunity. Our results suggest that overproduction of GM-CSF in AMs post-BMT leads to increased expression of EP2 and thus the exaggerated inhibition of AM function by PGE2 found post-BMT in the control BMT mice. Finding strategies to limit GM-CSF in the AMs, to optimize GM-CSF production in lung structural cells post-BMT, or to limit EP2 signaling (perhaps via an EP2 antagonist) in AMs may improve outcomes for HSCT patients.

This last recommendation would be consistent with previous studies that have demonstrated that EP2/–/– mice are protected from P. aeruginosa infection (45).

ACKNOWLEDGMENTS

Present address of R. Paine III: Dept. of Internal Medicine, Pulmonary Division, University of Utah, Salt Lake City, UT 84132.

GRANTS

This study was supported by National Institutes of Health Grant AI-065543 (B. B. Moore) and HL-058897 (M. Peters-Golden). M. N. Ballinger is supported by a postdoctoral fellowship from the Hartwell Foundation. L. L. N. Hubbard is supported by Research Training in Experimental Immunology Grant T32AI007413.

REFERENCES
