GM-CSF Regulates a PU.1-Dependent Transcriptional Program Determining the Pulmonary Response to LPS

Pierre-Yves Berclaz, Brenna Carey, Marie-Dominique Fillipi, Kara Wernke-Dollries, Nick Geraci, Stephanie Cush, Terry Richardson, Joe Kitzmiller, Michael O'Connor, Christina Hermoyian, Thomas Korfhagen, Jeffrey A. Whitsett, and Bruce C. Trapnell

Divisions of Pulmonary Biology and Experimental Hematology, Cincinnati Children's Hospital Medical Center; and University of Cincinnati College of Medicine, Cincinnati, Ohio

Alveolar macrophages (AMs) normally respond to lipopolysaccharide (LPS) by activating Toll-like receptor (TLR)-4 signaling, a mechanism critical to lung host defense against gram-negative bacteria such as *Pseudomonas aeruginosa*. Because granulocyte macrophage colony-stimulating factor (GM-CSF)-deficient (GM^{-/-}) mice are hyporesponsive to LPS, we evaluated the role of GM-CSF in TLR-4 signaling in AMs. Pulmonary TNF- α levels and neutrophil recruitment 4 h after intratracheal administration of *Pseudomonas* LPS were reduced in GM^{-/-} compared with wild-type (GM^{+/+}) mice. Secretion of TNF- α by AMs exposed to LPS *ex vivo* was also reduced in GM^{-/-} mice and restored in mice expressing GM-CSF specifically in the lungs (SPC-GM^{+/+}/GM^{-/-} mice). LPS-dependent NF- κ B promoter activity, TNF- α secretion, and neutrophil chemokine release were reduced in AM cell lines derived from GM^{-/-} mice (mAM) compared with GM^{+/+} (MH-S). Retroviral expression of PU.1 in mAM cells, which normally lack PU.1, rescued all of these AM defects. To determine whether GM-CSF, via PU.1, regulated expression of TLR-4 pathway components, mRNA and protein levels for key components were evaluated in MH-S cells (GM^{+/+}, PU.1^{Positive}), mAM cells (GM^{-/-}, PU.1^{Negative}), and mAMPU.1+ cells (GM^{-/-}, PU.1^{Positive}). Cluster of differentiation antigen-14, radioprotective 105, IL-1 receptor-associated kinase (IRAK)-M mRNA, and protein were dependent upon GM-CSF and restored by expression of PU.1. In contrast, expression of other TLR-4 pathway components (myeloid differentiation-2, TLR-4, IRAK-1, IRAK-2, Toll/IL-1 receptor domain containing adaptor protein/MyD88 adaptor-like, myeloid differentiation primary-response protein 88, IRAK-4, TNF receptor-associated factor-6, NF- κ B, inhibitor of NF- κ B kinase) were not GM-CSF or PU.1-dependent. These results show that GM-CSF, via PU.1, enables AM responses to *P. aeruginosa* LPS by regulating expression of a specific subset of components of the TLR-4 signaling pathway.

Keywords: alveolar macrophages; inflammation; lung; neutrophils

Pulmonary infection by *Pseudomonas aeruginosa* is a common complication in clinical settings associated with impaired airway clearance, for example, in cystic fibrosis and other bronchiectatic disorders or during periods of prolonged endotracheal intubation and mechanical ventilation. An intense pulmonary inflammatory response results, mediated in part by lipopolysac-

CLINICAL RELEVANCE

This study demonstrates that GM-CSF, via PU.1, enables the alveolar macrophage response to *Pseudomonas* LPS by stimulating expression of a specific subset of components of the Toll-like receptor 4 signaling pathway (CD14, IRAK-M, RP105).

charide (LPS), a component of the outer membrane of *Pseudomonas* and other gram-negative bacteria. Alveolar macrophages (AMs) play a central role in initiating the pulmonary inflammatory cascade and respond to LPS via activation of the Toll-like receptor (TLR)-4 signaling pathway (1). Soluble LPS-binding protein facilitates the delivery and binding of LPS to the TLR-4 receptor complex consisting of cluster of differentiation antigen 14 (CD14), myeloid differentiation (MD)-2, and TLR-4 proteins on the AM cell surface (2). The LPS-activated TLR-4 cell surface receptor complex signals through two distinct intracellular pathways: one involving binding and activation of the adapter molecule, myeloid differentiation primary-response protein 88 (MyD88) (3); and a second MyD88-independent pathway (4). Activated MyD88 signals sequentially through IL-1 receptor (IL-1R)-associated kinase 4 (IRAK-4) and IRAK-1, which become associated after activation. Subsequently, IRAK-1 is released and then activates TNF-associated factor-6 (TRAF-6). TRAF-6 then activates, via the adaptor proteins TAK-1, TAB-1, and TAB-2, several transcription factors, including NF- κ B and activator protein 1 (AP1). The MyD88-independent TLR-4 signaling pathway requires other adapter proteins such as Toll/IL-1R (TIR) domain containing adapter protein (TIRAP), TIR-domain-containing adaptor protein inducing IFN- β (TRIF), and TRIF-related adaptor molecule (TRAM) (4). Although these adaptors are essential for MyD88-independent TLR-4 signaling, the components of their signaling pathways are less well defined than the MyD88-dependent pathway (4). Nevertheless, this pathway also converges on NF- κ B and AP1, which activates genes resulting in synthesis of potent proinflammatory mediators such as TNF- α , type I interferons (IFN- α/β), proteases, and neutrophil chemokines that cause pulmonary neutrophil accumulation. These signaling pathways are also negatively regulated at a multiple points. For example, IRAK-M, which is restricted to monocytes and macrophages and is activated after stimulation with TLR ligands, appears to block MyD88-dependent TLR-4 signaling by preventing the dissociation of the IRAK-1-IRAK-4 complex, thus preventing IRAK-1 from binding and activating TRAF-6 (5). Radioprotective (RP) 105 is another molecule inhibiting the TLR-4 signaling pathway, which functions by inhibiting LPS binding to the TLR-4-MD2 complex (6).

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a 23-kD glycoprotein critical in surfactant homeostasis

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Correspondence and requests for reprints should be addressed to Bruce C. Trapnell, M.D., Children's Hospital Medical Center, Division of Pulmonary Biology, 3333 Burnet Avenue, Cincinnati, OH 45229. E-mail: Bruce.Trapnell@chmcc.org

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and lung host defense (7–9). In mice deficient in GM-CSF due to targeted gene ablation (GM^{-/-} mice), AM-mediated surfactant clearance and lung host defense are severely impaired (10–12). The defect in host defense is manifested by reduced pulmonary clearance of various microbial pathogens, including group B *Streptococcus* (13), *Pneumocystis carinii* (14), and *Mycobacterium tuberculosis* (15). *In vivo* studies in mice in which GM-CSF is expressed normally (GM^{+/+}), not at all (GM^{-/-}), or specifically in the lungs (SPC-GM^{+/+}/GM^{-/-}) demonstrated that maintenance of normal surfactant homeostasis and lung host defense requires the presence of GM-CSF in the lung (13, 16). *In vivo* studies in GM^{-/-}, SPC-GM^{+/+}/GM^{-/-}, and wild-type (GM^{+/+}) mice have shown that PU.1 expression in AMs is dependent on pulmonary GM-CSF (7). *In vitro* studies using an AM cell line derived from GM^{-/-} mice (mAM cells) identified a defect in AM-mediated TNF- α secretion after exposure to LPS derived from *Salmonella* (7). This defect was corrected by retroviral vector-mediated expression of PU.1, a transcription factor required to stimulate AM terminal differentiation (7). Together, these observations suggest that GM-CSF, via PU.1, may regulate lung inflammation by modulating the capacity of AMs to release proinflammatory cytokines and neutrophil chemotaxins after activation by microbial pathogens or products.

In this study we examined the role of GM-CSF and PU.1 in AM-mediated inflammatory host responses to *Pseudomonas* LPS *in vivo* and *in vitro*. Results show that GM-CSF is an important determinant of the capacity of AM proinflammatory immune responses following acute LPS exposure. GM-CSF, via PU.1, enabled TLR-4 signaling in AMs by increasing expression of a subset of the signaling components of the TLR-4 response system that includes CD14, RP105, and IRAK-M.

MATERIALS AND METHODS

Mice

C57BL/6, HEJ, and SNJ mice were from Charles River, Inc. (Wilmington, MA). MyD88 gene-ablated (MyD88^{-/-}) and strain-matched control (MyD88^{+/+}) mice were a generous gift from Shizuo Akira (Osaka University, Osaka, Japan). GM-CSF gene-ablated mice were previously created (11), and bred into and maintained in the C57BL/6 background (referred to as GM^{-/-} mice hereafter) (17). GM^{-/-} mice carrying a transgene that selectively expresses GM-CSF in the lungs from a human surfactant protein C promoter (SPC) were previously created (18) and bred into and maintained in the C57BL/6 background (referred to as SPC-GM^{+/+}/GM^{-/-} mice hereafter). C57BL/6 mice (referred to as GM^{+/+} hereafter) were used as the wild-type control for comparisons with both GM^{-/-} and SPC-GM^{+/+}/GM^{-/-} mice. All mice were housed in a barrier facility and studied under procedures approved by the Institutional Animal Care and Use Committee. Sentinel mice were tested periodically and were free of known viral and bacterial pathogens.

Alveolar Macrophages and Alveolar Macrophage Cell Lines

Primary AMs were obtained from mice by bronchoalveolar lavage (BAL) as described (7). MH-S (CRL-2019; American Type Culture Collection, Manassas, VA) is an AM cell line, previously derived from GM^{+/+} mice, with morphologic features and functions of normal, mature AMs (19). mAM is an AM cell line, previously derived from GM^{-/-} mice, with an incompletely differentiated phenotype due to absence of expression of the transcription factor PU.1. mAM cells constitutively expressing murine PU.1 were previously created by retroviral transduction of mAM cells (7). These cells also expressed green fluorescent protein (GFP), which was included in the vector as a selectable marker (20). As a transduction control, mAM cells expressing only the GFP marker were used (referred to as mAM hereafter) (7). Cultured AM cell lines (MH-S [GM^{+/+}, PU.1^{Positive}], mAM [GM^{-/-}, PU.1^{Negative}], and mAMPU.1+ [GM^{-/-}, PU.1^{Positive}]) were maintained as previously described (7). For readability, AMs obtained from genetically modified

mice will be referred to as primary AMs, and AM cell lines derived from genetically modified mice will be referred to as cultured AMs.

Pseudomonas LPS-Stimulated Pulmonary Inflammation

Mice underwent induction and maintenance of general anesthesia at moderate depth with Isoflurane (Forane; Ohmeda Caribe Inc., Liberty Corner, NJ) by titration to unconsciousness and mild bradypnea. After topical ethanol sterilization, a ventral midline neck incision was made and the trachea was minimally exposed and cannulated with a sterile 27-gauge needle/syringe. PBS containing LPS, purified from *Pseudomonas aeruginosa* 10 (hereafter *Pseudomonas* LPS, or LPS, purified by gel filtration chromatography and containing < 1% protein; reconstituted in PBS at a concentration of 25 ng/100 μ l, Catalog no. L8643; Sigma; St. Louis, MO;), was then slowly injected into the lungs. The needle was then withdrawn, the incision closed with surgical adhesive and the mice were allowed to recover in a sterile cage.

Pulmonary Neutrophil Recruitment

Four hours after LPS injection, mice were killed by lethal pentobarbital injection and bronchoalveolar lavage (BAL) was performed as described (7). BAL cells were sedimented by low-speed centrifugation (450 $\times$ g, 10 min, 4°C) and the BAL fluid was transferred to a sterile tube and frozen at -80°C until use. Cells were washed with PBS and sedimented onto polylysine-coated glass slides by cytocentrifugation (speed = 300, 3 min; Shandon, Pittsburgh, PA). Slides were stained with Diff Quik (Dade Diagnostics, Deerfield, IL) and the percentage of neutrophils in total BAL cells was determined by differential cytometric counting as previously described (21).

Enzyme-Linked Immunoabsorbant Assay

Frozen BAL samples were thawed and TNF- α protein concentration was quantified by enzyme-linked immunosorbent assay (ELISA) (murine Quantikine kit; R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. Cytokine release by LPS-stimulated AMs in culture was also quantified by ELISA. Primary AMs or cultured AMs were seeded into plastic culture dishes (200,000 cells/well, 12 well plate; Falcon; BD Biosciences, Bedford, MA). Four hours (primary AMs), or 24 h (cultured AMs) later, *Pseudomonas* LPS (Sigma) was added to a final concentration of 1 μ g/ml and cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 4 h the culture media was collected, cleared of cells by low-speed centrifugation as above, and the supernatant was evaluated by ELISA as described above to quantify TNF- α or MIP-1 α (murine Quantikine kit; R&D Systems).

Neutrophil Chemotaxis Assay

AMs were seeded into dishes and exposed to *Pseudomonas* LPS as above, and 4 h later medium was collected, cleared by centrifugation, and stored at -80°C until use. To quantify neutrophil chemotactic activity, cleared supernates were thawed and aliquots (23 μ l) were mixed with an aliquot (27 μ l) of Hanks' Balanced Salt Solution (with Ca²⁺ and Mg²⁺; HBSS). Fifty microliters of HBSS containing AM supernate, HBSS alone (as a negative control), or HBSS containing fMLP (1 μ M; as a positive control) was then placed in the lower chamber of 48-well micro-chemotaxis chamber plates (Neuro Probe, Gaithersburg, MD). HBSS containing neutrophils (2 $\times$ 10⁶ in 50 μ l) were placed in the upper compartments of the micro-chemotaxis chamber plates. The upper and lower wells were separated by a 3- μ m pore size polycarbonate filter (Poretics Products, Livermore, CA) that permits migration of neutrophils but not macrophages. The chamber plates were then incubated at 37°C for 60 min. After incubation, the upper side of each filter chamber was wiped clean to remove cells that had not migrated through the filter, and the filter was fixed and stained with Diff Quik. The number of neutrophils transmigrating through the filter was then counted on the lower surface of the filter. The mean number of neutrophils for four randomly chosen fields per filter and two filters per condition were determined.

NF- κ B Activation Assay

Transcription assays were performed essentially as described (22) except that Effectene (Qiagen, Valencia, CA) was used instead of a

calcium phosphate method and a renilla luciferase reporter was used as the internal transfection control instead of β -galactosidase. One day after plating, cells (2×10^5 /35-mm dish) were transfected with ELAM-LUC, an NF- κ B promoter-firefly luciferase reporter derived from reporter plasmid pGL3 (Promega, Inc., Madison, WI). Twenty-four hours later, transfected cells were treated with LPS (100 ng/well). Twenty-four hours after LPS activation, firefly and Renilla luciferase activities were quantified using a Monolight 3010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI) using the dual luciferase assay kit (Promega). Firefly luciferase values were normalized for Renilla luciferase activity. Data shown represent the mean of at least five determinations for each cell line.

RT-PCR Amplification

Specific mRNA transcripts were quantified in cultured AMs using RT-PCR as previously described (7, 21). Oligonucleotide primers used here for detection of TLR-4 signaling pathway component mRNAs are indicated (Table 1).

Flow Cytometry

Expression of RP105 was evaluated by flow cytometry as previously described (9). Briefly, cells were immunostained with either phycoerythrin (PE)-labeled rat anti-murine RP-105 antibody (12-1801-81) or PE-labeled rat IgG2a isotype control (both from eBioscience, San Diego, CA) according to the manufacturer's protocol and evaluated by flow cytometry (FACSscan). The difference in mean fluorescence, determined from the FL-2 channel, between anti-RP-105 and isotype control-stained samples was determined in triplicate and expressed as the mean $\pm$ SEM.

Western Blotting

Western blotting was performed as previously described (6). Briefly, primary antibodies included rat anti-murine CD14 (PharMingen, San Diego, CA) diluted 1:200, rabbit anti-mouse IRAK-M (Chemicon International, San Diego, CA) diluted 1:1,000, or goat anti-murine β -actin antibody (Santa Cruz, Santa Cruz, CA) diluted 1:200. Secondary antibodies, each of which were conjugated to horseradish peroxidase, included rabbit anti-rat IgG (Sigma) diluted 1:8,000, goat anti-rabbit IgG (Calbiochem, Temecula, CA) diluted 1:6,666, and rabbit anti-goat IgG (Sigma) diluted 1:7,000. Cellular protein lysates were electrophoresed on a 10–20% tris-glycine polyacrylamide gel (Invitrogen) and transferred to 0.2- μ M nitrocellulose membranes (BioRad Laboratories, Hercules, CA), blocked in 10% dry milk (4°C, overnight), and incubated with primary antibody at the indicated dilutions (4°C, overnight). After washing four times with PBS containing 0.1% Tween-20, membranes were incubated with respective secondary antibodies (room temperature, 45 min). Immunoreactive proteins were visualized by enhanced chemiluminescence (Amersham, Piscataway, NJ) and exposed to

Kodak X-Omat AR film (Kodak, New Haven, CT). Western blots were repeated five (CD14) or three (IRAK-M) times with similar results.

Statistics

All numeric data are presented as mean $\pm$ SEM. Statistical analysis was done using Sigma Stat (version 3.0) software (RockWare, Golden, CO) on an IBM-compatible microcomputer. Normality was evaluated by the Kolmogorov-Smirnov method and equal variance by the Levene median test. Comparisons between groups of normally distributed data of equal variance were made using one-way ANOVA with *post hoc* analysis by the Holm-Sidak method for comparisons between multiple groups. Nonparametric comparisons were made using Kruskal-Wallis one-way ANOVA on ranks with *post hoc* analysis by Dunn's method for multiple group comparisons.

RESULTS

LPS-Stimulated Pulmonary Neutrophil Recruitment Is Impaired in the Absence of Pulmonary GM-CSF

GM $^{-/-}$ mice have defects in lung host defense and AM immune functions. To determine if GM $^{-/-}$ mice also had defects in pulmonary neutrophil recruitment, LPS derived from *P. aeruginosa* was administered intratracheally to GM $^{+/+}$ or GM $^{-/-}$ mice, and 4 h later the percentage of neutrophils in the BAL fluid recovered was determined by differential cytometry. While LPS caused the rapid accumulation of pulmonary neutrophils in GM $^{+/+}$ mice, neutrophil accumulation was significantly less in GM $^{-/-}$ mice ($68.2 \pm 3.5\%$ versus $23.1 \pm 4.2\%$ of BAL cells, respectively; $n = 10$ /group, $P < 0.001$ [Holm-Sidak], Figure 1). Neutrophils comprised $< 1\%$ of BAL cells in unexposed GM $^{+/+}$ and GM $^{-/-}$ mice (not shown). These results demonstrate that GM-CSF is required for normal neutrophil recruitment after intratracheal LPS exposure.

GM-CSF Regulates LPS-Stimulated Proinflammatory Cytokine Release by AMs *In Vivo* and *In Vitro*

The role of GM-CSF in the acute proinflammatory cytokine signaling response of AMs *in vivo* was evaluated by quantifying TNF- α levels in BAL fluid from GM $^{+/+}$, GM $^{-/-}$, and SPC-GM $^{+/+}$ /GM $^{-/-}$ mice 4 h after intratracheal LPS administration. LPS caused a marked increase in pulmonary TNF- α levels in GM $^{+/+}$ mice and a smaller increase in GM $^{-/-}$ mice ($3,201 \pm 313$ versus $2,243 \pm 167$ pg/ml BAL; $n = 5$ /group, $P = 0.05$ [Holm-Sidak]; Figure 2A). In SPC-GM $^{+/+}$ /GM $^{-/-}$ mice, which overexpress GM-CSF in respiratory epithelial cells, LPS-stimulation of

TABLE 1. OLIGONUCLEOTIDE PRIMERS FOR DETECTION OF TLR-4 SIGNALING PATHWAY COMPONENT EXPRESSION BY RT-PCR

TLR-4 Signaling Component	Upstream RT-PCR Oligonucleotide Primer	Downstream RT-PCR Oligonucleotide Primer
TLR-4	5'-TACTCGAGTCAGAAATGAGGACTGG-3'	5'-TTCGAGGCTTTTCCATCCAATAGG-3'
CD-14	5'-CAGCTAAACTCGCTCAATCTGTC-3'	5'-ACTACGCCAGGTTAACTTCTCC-3'
MD-2	5'-GCCGAAGCGTAAGGAAGTCT-3'	5'-GCTTTTCTTCAGTATCCCCAGC-3'
RP105	5'-TCTGGACCTGGGATGGCTCC-3'	5'-AATGTGTTGGTATTCCATCTGAGA-3'
TOLLIP	5'-AAGCCTCGAGACAAGATAGCTGAC-3'	5'-AGGTAGGTGACATTCTCTG-3'
TIRAP	5'-CCGTATAACACCCACACACAG-3'	5'-GAGTTTTCATAAACAGCATAGCC-3'
IRAK-1	5'-GGTTCACCTCCCTGTTCC-3'	5'-AAACCACCTCTCCAATCCT-3'
IRAK-2	5'-TCATCCCAGCAATAAAAGACC-3'	5'-AATCTCACTGAGAAGCAATCC-3'
IRAK-4	5'-ACTTCTCTGCAAGCTCTTCTC-3'	5'-TCCCTGTCTCCTCTGTTTC-3'
IRAK-M	5'-ACTCCTCAAGCCCTCAGACAAC-3'	5'-TCATGCAGAACTGATGCC-3'
TRAF-6	5'-TGTGTAAGTGTGCTGTGTC-3'	5'-TCCCAACTTGCCAATCTTC-3'
IKKB	5'-GTGTGACGTGTATCCTTC-3'	5'-GCTCCACAGCCTGCTCC-3'
NF- κ B	5'-CATTCTGACCTTGCTATCTAC-3'	5'-CCCCAAATCCTTCCCAAC-3'
GAPDH	5'-ATTCTACCCACGGCAAGTTC-3'	5'-AGGGGCGGAGATGATGACCC-3'

Definition of abbreviations: CD-14, cluster of differentiation antigen-14; GAPDH, glyceraldehyde phosphate dehydrogenase; IKKB, inhibitor of NF- κ B kinase; IRAK, IL-1 receptor-associated kinase; MD-2, myeloid differentiation 2; RP105, radioprotective 105; TIRAP, Toll/IL-1 receptor domain containing adapter protein; TLR-4, Toll-like receptor-4; TRAF-6, TNF-associated factor 6.

Figure 1. GM-CSF deficiency impairs LPS-stimulated pulmonary neutrophil recruitment. Four hours after intratracheal *Pseudomonas* LPS administration, pulmonary neutrophil influx determined by differential cytometry was reduced in GM-CSF-deficient (GM^{-/-}) mice compared with wild-type (GM^{+/+}) controls ($n = 10$, $P < 0.001$ [ANOVA]).

pulmonary TNF- α was increased compared with wild-type mice ($4,617 \pm 291$ pg/ml BAL; $n = 5$, $P < 0.001$ [Holm-Sidak]; Figure 2A). In the absence of LPS, TNF- α levels were < 12 pg/ml in BAL in all three mouse lines (not shown). To determine if these differences were caused by a direct effect of GM-CSF on AMs, primary AMs from each mouse line were stimulated by LPS *ex vivo* and TNF- α release was quantified by ELISA. Similar to the *in vivo* results, LPS increased TNF- α secretion from wild-type (GM^{+/+}) AMs and SPC-GM^{+/+}/GM^{-/-} AMs, and was less active in GM^{-/-} mice ($4,707 \pm 375$ versus $6,283 \pm 417$ versus 999 ± 106 pg/ml, respectively; $n = 5$ /group, $P < 0.01$ [all comparisons, Holm-Sidak]; Figure 2B).

LPS-stimulated TNF- α secretion was also evaluated in AM cell lines derived from GM^{+/+} mice (MH-S cells) and GM^{-/-} mice (mAM cells). LPS stimulated secretion of TNF- α from MH-S cells but not from mAM cells ($3,797 \pm 62$ versus 0 ± 0

Figure 2. GM-CSF deficiency impairs *Pseudomonas* LPS-stimulated TNF- α release by AMs *in vivo* and *in vitro*. (A) Levels of TNF- α in the BAL fluid recovered 4 h after pulmonary administration of LPS were reduced in GM^{-/-} mice compared with GM^{+/+} controls and were restored to supranormal levels in BAL from GM^{-/-} mice carrying a transgene overexpressing GM-CSF in the lungs (SPC-GM^{+/+}/GM^{-/-} mice; $n = 5$, $P < 0.001$ [ANOVA]). (B) TNF- α secretion by primary AMs after recovery from BAL fluid, adherence to plastic for 1 h and exposure to LPS for 4 h. TNF- α secretion was reduced in AMs from GM^{-/-} mice compared with GM^{+/+} controls and was restored to supranormal levels in cells from SPC-GM^{+/+}/GM^{-/-} mice ($n = 5$, $P = 0.001$ [ANOVA]). (C) TNF- α secretion by cultured murine AM cell lines after exposure to LPS

for 4 h. TNF- α secretion was readily detectable in MH-S (GM^{+/+}) macrophages, absent in mAM (GM^{-/-}) macrophages (asterisk) and was restored to supranormal levels in mAMPU.1+ (GM^{-/-}, PU.1^{Positive}) macrophages by retroviral-mediated overexpression of the transcription factor PU.1 ($n = 5$, $P < 0.001$ [Kruskal-Wallis ANOVA on ranks]).

pg/ml media, respectively, $n = 6$ /group; Figure 2C). Retroviral expression of PU.1 in mAM cells (mAMPU.1+ cells) restored TNF- α responses to *Pseudomonas* LPS ($6,057 \pm 62$ pg/ml, $n = 6$; Figure 2C). These data show that GM-CSF regulates LPS-stimulated TNF- α expression in the lung *in vivo* and in AMs *ex vivo*. PU.1 bypassed the requirement for GM-CSF and restored TNF- α secretion by AMs in response to *Pseudomonas* LPS.

GM-CSF, via PU.1, Regulates LPS-Stimulated Neutrophil Chemotactic Activity by AMs

Since activated AMs release potent neutrophil chemotactic factors, we evaluated the role of GM-CSF and PU.1 in regulating release of neutrophil chemotactic activity in LPS-stimulated AM cell lines using a standard Boyden chamber assay. LPS increased neutrophil chemotactic activity release from MH-S and mAMPU.1+ cells compared with the respective unstimulated cells (206 ± 63 versus 63 ± 4 , and 204 ± 12 versus 74 ± 5 neutrophils per high-power field [hpf], respectively; $n = 9$, $P < 0.05$ [Dunn's]; Figure 3A). In contrast, LPS did not stimulate release of chemotactic activity in mAM cells (60 ± 6 versus 63 ± 3 neutrophils/hpf, with and without LPS, respectively, $n = 9$; Figure 3A).

To determine if GM-CSF-induced chemotactic activity was cytokine in nature, the culture supernatants of LPS-stimulated and unstimulated AM cell lines was evaluated for the presence

Figure 3. Neutrophil chemotaxis release from *Pseudomonas* LPS-stimulated AMs is regulated by GM-CSF, via PU.1. (A) Quantification of neutrophil chemotactic activity for wild type neutrophils in a Boyden chamber. AM cell lines were exposed to LPS for 24 h and culture supernatant was collected and evaluated for the presence of neutrophil chemotactic activity. LPS-exposure stimulated secretion of neutrophil chemotactic activity from MH-S cells (GM^{+/+}) but not mAM cells (GM^{-/-}) and was restored in mAMPU.1+ cells (GM^{-/-}, retroviral PU.1+) ($n = 9$ /group; $P < 0.001$ [Kruskal-Wallis ANOVA on ranks]). As controls, fMLP stimulated readily increased neutrophil chemotaxis compared with media alone (hatched and open bars, respectively; $n = 3$ /group, $P < 0.001$). (B) Quantification of LPS-stimulated MIP-1 α secretion. Cell lines were exposed to LPS as above and MIP-1 α was quantified in culture supernatant by ELISA. LPS stimulated significantly increased MIP-1 α secretion in MH-S and mAMPU.1+ cells, but did not stimulate any increase in mAM cells ($n = 4$ /group, $P < 0.001$ [Kruskal-Wallis ANOVA on ranks]). * indicates "not detected".

of MIP-1 α . LPS stimulated release of MIP-1 α from MH-S but not mAM cells and was restored in mAMPU.1+ cells (Figure 3B). Thus, PU.1 is required for release of MIP-1 α by LPS-stimulated AMs.

GM-CSF Regulates TLR-4-Dependent Responses to *Pseudomonas* LPS

The role of TLR-4 receptor signaling in the *Pseudomonas* LPS response was assessed in SNJ mice, in which the TLR-4 molecule is intact, and in HEJ mice, in which TLR-4 is nonfunctional (2). Intrapulmonary administration of LPS caused robust pulmonary neutrophil recruitment in SNJ mice and a lesser response in HEJ mice (73 ± 4 versus 26 ± 8 cells/hpf; $n = 9$, $P < 0.001$ [Holm-Sidak]; Figure 4A). Pulmonary TNF- α levels were significantly lower in HEJ compared with SNJ mice 4 h after LPS administration ($1,983 \pm 296$ versus $8,478 \pm 851$ pg/ml BAL, respectively, $n = 15$; $p < 0.001$ [Holm-Sidak]; Figure 4B).

To determine the role of MyD88 in the pulmonary inflammatory response to *Pseudomonas* LPS, AMs from MyD88 $^{-/-}$ mice and controls were treated with LPS *ex vivo*. Secretion of neutrophil chemotactic activity by AMs was evaluated by Boyden chamber assay. LPS-exposed AMs from MyD88 $^{+/+}$ mice secreted significantly more neutrophil chemotactic activity compared with LPS-exposed AMs from MyD88 $^{-/-}$ mice (198 ± 8 versus 59 ± 3 cells/hpf, $n = 3$; $P < 0.001$ [Holm-Sidak]; Figure 4C). As a positive control, fMLP stimulated chemotaxis of GM $^{+/+}$ neutrophil used in the assay (212 ± 7 cells/hpf, $n = 3$).

NF- κ B transcriptional activity was assessed in cultured AM cell lines using a NF- κ B-responsive promoter coupled to a luciferase reporter. In MH-S cells, LPS stimulated NF- κ B promoter activity 150 ± 43 -fold above that of unexposed cells ($n = 24$). In contrast, the effect of LPS stimulation in mAM cells was decreased 7 ± 1 -fold ($n = 18$; $p < 0.05$ [Dunn's]; Figure 4D). Expression of PU.1 in mAMPU.1+ cells restored LPS-stimulated NF- κ B promoter activity to 223 ± 43 -fold that of unexposed mAMPU.1+ cells ($n = 20$; $p < 0.05$ [Dunn's]; Figure 4D).

These results suggest that GM-CSF, via PU.1, regulates molecular and cellular components of the pulmonary inflammatory response to *Pseudomonas* LPS by modulating the TLR-4 signaling in AMs. To evaluate this hypothesis, we quantified mRNA levels for proteins known to comprise components of the TLR-4 signaling pathway in AMs by RT-PCR analysis. MH-S and mAMPU.1+ cells contained mRNA transcripts for TLR4, CD-14, MD-2, RP105, Tollip, TIRAP, IRAK-1, IRAK-2, IRAK-4, IRAK-M, TRAF-6, inhibitor of NF- κ B kinase (IKKB), and NF- κ B (Figure 5). In contrast, mAM cells contained mRNA transcripts for each of these factors, but were lacking CD-14, RP105, and IRAK-M (Figure 5). These results were confirmed by quantification of protein levels for these three factors in AM cell lines. RP105 was detected in MH-S cells, absent in mAM cells and restored by PU.1 expression in mAMPU.1+ cells as shown by flow cytometry (Figures 6A and 6B). A similar pattern was found for CD14 and IRAK-M as shown by Western analysis (Figure 6C). Thus, GM-CSF, via PU.1, coordinately regulates expression of multiple molecular components of the TLR-4 signaling pathway in AMs, including both positive (CD14) and negative (RP105, IRAK-M) signaling components.

DISCUSSION

This study demonstrates that GM-CSF plays a critical role in modulating pulmonary inflammation by regulating the capacity of AMs to secrete proinflammatory cytokines and neutrophil chemokines in response to *P. aeruginosa* LPS. GM-CSF, via PU.1, stimulates a transcriptional program in AMs, enabling

Figure 4. GM-CSF regulates *Pseudomonas* LPS-stimulated neutrophil recruitment via the TLR-4 signaling pathway. (A) The percentage of neutrophils in cells recovered by BAL 4 h after pulmonary administration of LPS was reduced in HEJ mice (TLR-4 deficient) compared with SNJ (TLR-4-replete) ($n = 9$, $P < 0.001$ [ANOVA]). (B) Levels of TNF- α in the BAL fluid recovered 4 h after pulmonary administration of LPS were reduced in HEJ mice compared with SNJ controls ($n = 15$, $P < 0.001$ [ANOVA]). (C) Neutrophil chemotaxis release by primary AMs after exposure to LPS for 24 h. Neutrophil chemotaxis release by macrophages from MyD88-deficient (MyD88 $^{-/-}$) mice was reduced compared with wild-type controls (MyD88 $^{+/+}$) ($n = 3$, $P < 0.001$ [ANOVA]) as measured by the Boyden chamber assay. As a positive control, fMLP stimulated neutrophil chemotaxis ($n = 3$, hatched bars). (D) LPS-stimulated NF- κ B activity was markedly reduced in cultured alveolar macrophages from GM $^{-/-}$ mice (mAM) compared with GM $^{+/+}$ mice (MH-S) and was restored by expression of PU.1 in GM $^{-/-}$ cells (mAMPU.1+) ($n = 20$, $P < 0.001$ [ANOVA]).

Figure 5. GM-CSF, via PU.1, regulates mRNA levels for multiple components of the TLR-4 signaling pathway in AMs. mRNA transcripts encoding both positive and negative signaling components of the TLR-4 signaling pathway in cultured alveolar macrophages were detected by RT-PCR. Note the absence of transcripts for CD14, RP105, and IRAK-M in mAM cells.

the TLR-4 signaling pathway. In AMs, PU.1 is required for expression of some TLR-4 signaling pathway components (CD14, RP105, IRAK-M), while expression of others (TLR-4, MD2, MyD88, IRAK-1, TIRAP, IRAK-2, IRAK-4, TRAF-6, IKKB, NF- κ B, Tollip) appears to be minimally or unaffected by GM-CSF or PU.1. These results have implications for mechanisms of pulmonary host defense and potential clinical strategies to

Figure 6. GM-CSF, via PU.1, regulates multiple components of TLR-4 signaling pathway in AMs. (A) Evaluation of RP105 on cultured alveolar macrophage cell lines by flow cytometry. Cells were immunostained with PE-labeled, anti-RP105 (black line) or isotype control (gray shading) antibodies and evaluated by flow cytometry. (B) RP105 levels on AMs were quantified by determining the difference between the mean fluorescence of respective cell lines immunostained with anti-RP105 antibody minus the isotype control antibody. RP105 was readily detectible on MH-S and mAMPU.1+ cells and absent on mAM cells ($n = 3$ determinations/cell line; $P < 0.001$ [Kruskal-Wallis ANOVA on ranks]). (C) Evaluation of CD14 and IRAK-M on cultured alveolar macrophage cell lines by Western blot analysis.

modulate pulmonary inflammation via pharmacologic regulation of GM-CSF bioactivity.

Our data support a model in which GM-CSF confers LPS responsiveness to AMs by coordinately stimulating the expression of multiple components of the TLR-4 signaling pathway (Figure 7). While GM-CSF does not directly activate TLR-4 signaling in AMs, GM-CSF enables the *capacity* for signaling by enhancing expression of molecules required for TLR-4 signaling (CD14) or that negatively regulate TLR-4 signaling (RP105, IRAK-M). This type of global regulation of positive and negative elements of the pathway is similar to regulation of AM Fc receptor signaling by GM-CSF. In the Fc receptor pathway, GM-CSF (via PU.1) also regulates the expression of both positive (Fc γ RIA, Fc γ RIIIA) and negative (Fc γ RIIB) receptors (8) as well as other pathway components (FcR common γ chain, Syk and Hck; P.-Y. Berclaz and B. C. Trapnell, unpublished observations). The proposed model is consistent with our prior observations (7) and provides a molecular explanation for the increased endotoxin tolerance that has been reported to occur in GM- $^{-/-}$ mice (23) and is consistent with a report that administration of anti-GM-CSF antibodies to mice reduces LPS-stimulated lung inflammation (24). Finally, the coordinate regulation of both positive and negative regulatory components is consistent with

Figure 7. Proposed mechanism by which GM-CSF regulates AM-mediated proinflammatory cytokine signaling and neutrophil recruitment upon exposure to *Pseudomonas* LPS. GM-CSF receptor binding stimulates increased levels of the transcription factor, PU.1, in AMs (see Ref. 6). In turn, PU.1 stimulates expression of TLR-4 signaling pathway components (arrows). Expression of other TLR-4 components in alveolar macrophages is unaffected by GM-CSF or PU.1 (unmarked).

the concept that GM-CSF upregulates TLR-4 signaling as part of a program regulating terminal differentiation of AMs.

The observation that GM-CSF is critical for AM release of proinflammatory cytokines, neutrophil chemotaxins, and the neutrophil-dominated inflammatory response to intrapulmonary LPS injury has important implications for lung host defense. GM^{-/-} mice have increased spontaneous mortality due to pulmonary and systemic infections (25) and increased susceptibility to bacterial (13), fungal (14), and mycobacterial (15) pathogens. AMs from these mice also have defects in clearance of microbial pathogens, particles, and surfactant (reviewed in Ref. 26). The GM^{-/-} mouse has a human parallel in a rare but fascinating disorder known as pulmonary alveolar proteinosis (27), in which high levels of neutralizing anti-GM-CSF autoantibodies appear to eliminate GM-CSF bioactivity *in vivo* (28) resulting in a functional GM-CSF deficiency. In this disorder, 18% of attributable mortality is reportedly due to uncontrolled infections (29). AMs from individuals with PAP have a number of abnormalities similar to those of AMs from GM^{-/-} mice including reduced expression of PU.1, CD14, FcγR and other receptors, and a reduced phagocytic capacity (7, 27, 30–34). Defects in AM function, including impaired secretion of inflammatory cytokines and chemokines, may contribute to the increase in infections observed in individuals with PAP and in GM^{-/-} mice. Our observations suggest that GM-CSF is critical for the AM response to intrapulmonary *Pseudomonas* LPS and may be relevant to cystic fibrosis, since *Pseudomonas* colonization of the airway is a common finding. This is supported by the observation that pulmonary GM-CSF levels are elevated in individuals with cystic fibrosis (35). In GM^{-/-} mice, the TNF-α response of AMs to LPS exposure *ex vivo* was severely impaired while the response to *in vivo* exposure was only mildly impaired, suggesting that LPS may activate TLR-4 signaling in another cell type, which is unaffected by GM-CSF/PU.1 deficiency. Consistent with this possibility, respiratory airway epithelial cells have been reported to express TLR-4 and undergo an inflammatory activation response after exposure to LPS (36). Another possibility is that LPS may signal through a pathway other than TLR-4. This is supported by our observation that the TNF-α response was not abolished in HEJ mice. A trivial explanation for the discrepancy is the potential presence of an LPS contaminant that signals through a second pathway not present in AMs. Although theoretically possible, this is unlikely because we used highly purified LPS isolated by molecular sieve gel filtration chromatography. While our results demonstrate that LPS-exposed AMs secrete MIP-1α, a potent neutrophil chemoattractant (37, 38), LPS stimulates macrophages to secrete multiple cytokines and chemokines (39), several of which participate in neutrophil recruitment.

GM-CSF appears to regulate the capacity of AMs to respond to LPS in a continuous fashion, that is, “rheostatically” rather than dichotomously (i.e., “off-on”). Our data supporting such a mechanism include that *Pseudomonas* LPS caused: (1) higher pulmonary TNF-α levels in mice overexpressing pulmonary GM-CSF (SPC-GM^{+/+}/GM^{-/-}) compared with wild-type (GM^{+/+}) mice; (2) greater TNF-α secretion by AMs from SPC-GM^{+/+}/GM^{-/-} compared with GM^{+/+} mice; (3) greater TNF-α secretion by AMs overexpressing PU.1 (mAMPU.1+) compared with wild-type (MH-S); and (4) greater NF-κB promoter activation in mAMPU.1+ compared with MH-S cells. Finally, the TNF-α response of primary AMs from GM^{-/-} mice was severely reduced, but not absent, in contrast to mAM cells, in which a TNF-α response was completely absent. These responses correlate with the respective levels of PU.1 expression in these cells; very low but not absent in the former and undetectable in the latter, even by real-time PCR (S. A. Abe and B. C. Trapnell, unpublished observations). It is possible that the very low levels

of PU.1 in GM^{-/-} AMs result in low levels of CD14 expression. In contrast, marked increased pulmonary GM-CSF levels result in overexpression of PU.1 in AMs (7). The concept of rheostatic control of TLR-4 signaling in AMs is consistent with a recent report demonstrating an inverse dose–response relationship between the amount of pulmonary anti-GM-CSF antibody administered and pulmonary neutrophil recruitment (24). Such a mechanism may have important therapeutic implications for strategies to reduce inflammation in inflammatory lung disorders such as asthma or cystic fibrosis. For example, anti-GM-CSF antibodies, or a small molecule equivalent, could reduce pulmonary GM-CSF bioactivity, thus lowering the degree of lung inflammation. Conversely, supplementation of pulmonary GM-CSF activity to augment AM effector and regulatory functions may be useful in the treatment of difficult pulmonary infections such as tuberculosis, for which pulmonary GM-CSF is an important determinant of the host response (15), and for which antibiotic therapy is increasingly complicated by drug-resistant organisms.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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