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GM-CSF Regulates Alveolar Macrophage Differentiation and Innate Immunity in the Lung through PU.1

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Summary

GM-CSF gene targeted ($GM^{-/-}$) mice are susceptible to respiratory infections and develop alveolar proteinosis due to defects in innate immune function and surfactant catabolism in alveolar macrophages (AMs), respectively. Reduced cell adhesion, phagocytosis, pathogen killing, mannose- and Toll-like receptor expression, and LPS- or peptidoglycan-stimulated TNFα release were observed in AMs from $GM^{-/-}$ mice. The transcription factor PU.1 was markedly reduced in AMs of $GM^{-/-}$ mice in vivo and was restored by selective expression of GM-CSF in the lungs of SPC-GM/$GM^{-/-}$ transgenic mice. Retrovirus-mediated expression of PU.1 in AMs from $GM^{-/-}$ mice rescued host defense functions and surfactant catabolism by AMs. We conclude that PU.1 mediates GM-CSF-dependent effects on terminal differentiation of AMs regulating innate immune functions and surfactant catabolism by AMs.

Introduction

The alveolar macrophage (AM) has a critical role in both lung host defense and surfactant homeostasis. AMs contribute to innate immunity in the lung by virtue of their ability to migrate, phagocytose, and kill microorganisms, and recruit and activate other inflammatory cells. Macrophages also provide important links between innate and adaptive immunity—for example, by Toll-like receptor-mediated pathogen recognition that results in release of cytokines capable of reprogramming other innate and adaptive immune responses (Aderem and Ulevitch, 2000). AMs are vital to normal lung surfactant metabolism, to which they contribute by catabolism of both surfactant lipids and surfactant proteins (Yoshida et al., 2001). Although AMs originate from hematopoietic stem cells in bone marrow (Thomas et al., 1976), they are predominantly derived from circulating blood monocytes (Kennedy and Abkowitz, 1998) which enter the lung and other tissues and differentiate into morphologically, histochemically, and functionally distinct tissue macrophage populations (Hume et al., 1983). The mechanisms mediating terminal differentiation of AMs are not known but are of fundamental significance because of the critical and diverse roles of these cells.

Granulocyte macrophage-colony stimulating factor (GM-CSF) promotes the survival, differentiation, proliferation, and function of myeloid progenitors (Rasko, 1994) as well as the proliferation and function of AMs (Akagawa et al., 1988; Chen et al., 1988) and pulmonary type II alveolar epithelial cells (Huffman Reed et al., 1997). An unexpected but critical role for GM-CSF in lung homeostasis was revealed by ablation of murine loci for GM-CSF ($GM^{-/-}$ mice) (Dranoff et al., 1994) or its receptor (GM $R\beta c^{-/-}$ mice) (Robb et al., 1995), both of which result in pulmonary alveolar proteinosis (PAP) and abnormalities of AM function. AM abnormalities in $GM^{-/-}$ mice include (1) surfactant accumulation (Dranoff et al., 1994) due to decreased catabolism of surfactant lipids and proteins by AMs (Ikegami et al., 1996; Yoshida et al., 2001); (2) defective phagocytosis of fungi (Paine et al., 2000); and (3) decreased oxygen radical production (LeVine et al., 1999). $GM^{-/-}$ mice have defective pulmonary clearance of bacterial or fungal pathogens and increased susceptibility and abnormal cytokine responses to lung infection (LeVine et al., 1999). These defects are corrected by pulmonary administration of GM-CSF or by expression of GM-CSF in the lungs either by somatic gene transfer or in transgenic mice utilizing a lung-specific transgene (SPC-GM/$GM^{-/-}$ mice) (Huffman et al., 1996; Reed et al., 1999; Zsengeller et al., 1998). In GM $R\beta c^{-/-}$ mice, correction of PAP by bone marrow transplantation suggested that disturbance of an effect of GM-CSF on AMs rather than type II alveolar epithelial cells is responsible for PAP in this model (Nishinakamura et al., 1996b). These data demonstrate that GM-CSF regulates diverse functions of AMs critical to surfactant homeostasis and host defense; however, the mechanism(s) by which this occurs are not known.

PU.1 is an *ets*-family transcription factor that regulates myeloid and B cell lineage development (Klemsz et al., 1990; Scott et al., 1994). PU.1 gene disruption abolishes macrophage and B lymphocyte production and delays neutrophils and T lymphocyte production (Scott et al., 1994; McKercher et al., 1996). Although not essential for myeloid lineage commitment (Olson et al., 1995), PU.1 promotes both the proliferation (Celada et al., 1996) and differentiation (DeKoter et al., 1998) of myeloid progenitors. Recently, GM-CSF and PU.1 have been proposed to have distinct roles in late-stage myeloid development, with GM-CSF promoting cell proliferation and PU.1 promoting differentiation (Anderson et al., 1999). To discern the role of GM-CSF and PU.1 in mechanisms regulating AM terminal differentiation and development of normal innate immune function in the lung, AMs from $GM^{-/-}$, $GM^{+/+}$, and SPC-GM/$GM^{-/-}$ mice were studied in vivo and in vitro. Results showed that GM-CSF stimulated PU.1 protein levels in AMs in vivo and that the absence of PU.1 in AMs strongly correlated with functional defects in AMs, including decreased surfactant catabolism, cell adhesion, phagocytosis, bacterial killing, pathogen recognition receptor expression, and inflammatory cytokine signaling. Importantly, retrovirus-medi-

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ated PU.1 expression in AMs of GM$^{-/-}$ mice rescued or stimulated all of these functions. We conclude that GM-CSF stimulates terminal differentiation of AMs predominately through PU.1 and, in so doing, regulates critical components of the innate immune system in the lung.

Results

GM-CSF Increases PU.1 Levels in AMs In Vivo

GM$^{-/-}$ and SPC-GM/GM$^{-/-}$ mice were generated previously (Dranoff et al., 1994; Huffman et al., 1996). GM-CSF is absent in GM$^{-/-}$ mice, while in SPC-GM/GM$^{-/-}$ mice GM-CSF is detectable at high levels in the lungs but not blood, (Huffman et al., 1996) and in GM$^{+/+}$ mice it is detectable in the lungs but only at low levels (Paine et al., 2000). For clarity and ease of presentation, AMs from various sources will be referenced by subscripts that indicate the relevant genetic manipulation of mice from which they were derived, i.e., AM$_{GM}^{+/+}$, AM$_{GM}^{-/-}$, and AM$_{SPC\text{-}GM/GM}^{-/-}$ designate primary AMs from GM$^{+/+}$, GM$^{-/-}$, and SPC-GM/GM$^{-/-}$ mice, respectively. Western analysis was used to assess the role of GM-CSF in regulating PU.1 levels in primary AMs (Figure 1A). PU.1 was readily detected in AM$_{GM}^{+/+}$, absent in AM$_{GM}^{-/-}$, and was rescued in AM$_{SPC\text{-}GM/GM}^{-/-}$, where levels were higher than wild-type controls. To obtain data from primary AMs completely free of extracellular alveolar proteinosis material, primary AMs were adhered to plastic dishes for 1 hr and gently but extensively washed prior to lysis for Western analysis. PU.1 was present in AM$_{GM}^{+/+}$, was increased in AM$_{SPC\text{-}GM/GM}^{-/-}$, and was present only in trace amounts in AM$_{GM}^{-/-}$ (Figure 1B). Thus, PU.1 was detected in primary AMs only in the presence of GM-CSF, and local GM-CSF levels in lung correlated with PU.1 levels in AMs. M-CSF was quantified by enzyme-linked immunosorbent assay (Shibata et al., 2001) to evaluate its potential role in AM terminal differentiation in this model. As expected, M-CSF was present in the lungs of GM$^{+/+}$ mice (Figure 1C). However, M-CSF levels were significantly elevated in the lungs of GM$^{-/-}$ mice compared to controls ($p < 0.001$) and normalized by lung-specific GM-CSF expression in SPC-GM/GM$^{-/-}$ mice. These results show that the differentiation defect (see below) in GM$^{-/-}$ mice is selectively restored by GM-CSF/PU.1 and not by M-CSF in SPC-GM/GM$^{-/-}$ mice. Together, these results demonstrate that local GM-CSF expression within the lung regulates PU.1 levels in AMs in vivo. We next evaluated the effect of forced PU.1 expression in AMs from GM$^{-/-}$ mice using an AM cell line (mAM) derived from GM$^{-/-}$ mice (Z.C.C., unpublished data). These cells were transduced with retroviral vectors expressing either PU.1 and green fluorescent protein ([GFP] as a selectable marker) or GFP alone (as a negative control) as previously described (DeKoter and Singh, 2000) to generate cell lines designated AM$_{GM}^{-/-}{}_{/PU.1}^{+}$ and AM$_{GM}^{-/-}{}_{/GFP}^{+}$, respectively. An AM cell line previously established from GM$^{+/+}$ mice (designated AM$_{GM}^{+/+}{}_{/MHS}$) with phagocytic and bacteriocidal activity typical of normal AMs (Mbawuike and Herscowitz, 1989) served as an additional positive control. PU.1 was expressed at high levels in AM$_{GM}^{+/+}{}_{/MHS}$ and also in AM$_{GM}^{-/-}{}_{/PU.1}^{+}$ but not in AM$_{GM}^{-/-}{}_{/GFP}^{+}$ (Figure 1A).

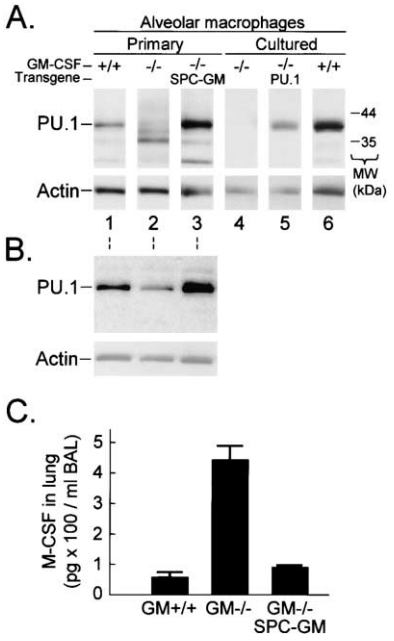

Figure 1. GM-CSF Regulates PU.1 Expression in Alveolar Macrophages

(A) PU.1 was assessed by Western blotting in primary alveolar macrophages (AM) from mice in which endogenous GM-CSF expression is normal (+/+, lane 1), absent due to gene targeting (−/−, lane 2), or expressed only in the lungs of GM-CSF gene-ablated mice from a lung-specific promoter-driven transgene (−/− SPC-GM, lane 3). PU.1 was also assessed in cultured AM cell lines established from GM$^{-/-}$ mice transduced with a control retroviral vector (−/−, lane 4) or with a PU.1-expressing vector (−/− PU.1, lane 5), or an AM cell line derived from wild-type (GM$^{+/+}$, lane 6) mice. Actin controls are shown.

(B) To eliminate alveolar proteinosis material from the preparations, primary AMs were adhered to plastic dishes for 1 hr prior to cell lysis for Western blotting analysis.

(C) M-CSF levels were quantified in unconcentrated lung lavage fluid of GM$^{+/+}$, GM$^{-/-}$, and SPC-GM/GM$^{-/-}$ mice (n = 8, 8, and 4, respectively) by ELISA.

Forced Expression of PU.1 in AMs from GM$^{-/-}$ Mice Rescues Macrophage Morphology and Cell Adhesion

Primary AM$_{GM}^{-/-}$ had a characteristic large, foamy appearance due to intracellular accumulation of surfactant lipids and protein in vivo, findings that were corrected in AM$_{SPC\text{-}GM/GM}^{-/-}$ (Figure 2A). Cultured AM$_{GM}^{-/-}{}_{/GFP}^{+}$ were large and flat with homogenously staining cytoplasm. Forced PU.1 expression rescued a macrophage-like phenotype consisting of a rounded shape, smaller size, and increased vacuolization, as seen in AM$_{GM}^{-/-}{}_{/PU.1}^{+}$. Morphometric analysis of cultured AMs revealed that PU.1 significantly reduced the cell diameter from 23.5 ± 0.5 to 14.5 ± 0.28 μm ($p < 0.00001$) (Figure 2B). Adherence was reduced in AM$_{GM}^{-/-}{}_{/GFP}^{+}$, consistent with the known effect of GM-CSF on primary myeloid cells (Arnaout et al., 1986), but was significantly increased by PU.1 ($p < 0.001$) (Figure 2C).

Expression of PU.1 in AMs from GM$^{-/-}$ Mice Rescues Surfactant Catabolism

To determine the role of PU.1 in surfactant catabolism by AMs, we assessed uptake and degradation of surfactant

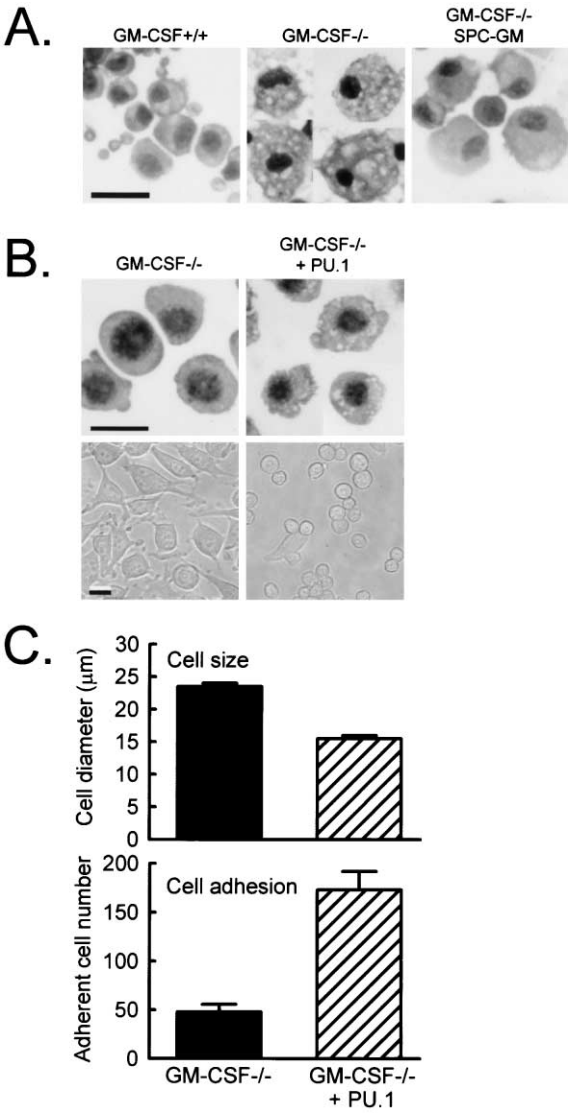

Figure 2. Rescue of a Normal AM Phenotype by GM-CSF Overexpression in the Lung In Vivo or Retrovirus-Mediated PU.1 Expression In Vitro

(A) Morphology of primary AMs from $GM^{+/+}$ mice (left), $GM^{-/-}$ mice (middle), and SPC-GM/$GM^{-/-}$ mice (right). Note the increased surfactant accumulation in $AM_{GM}{}^{-/-}$ but not $AM_{GM}{}^{+/+}$ or $AM_{SPC\text{-}GM/GM}{}^{-/-}$. Cytospin preparations were stained with Diff-Quick and evaluated by light microscopy. Original magnification is 100×; bar represents 20 μm.

(B) The top panels show morphology of cultured AMs from $GM^{-/-}$ mice after retroviral transduction with a control vector (left) or PU.1-expressing vector (right). Note increased vacuolization induced by PU.1. Cells were collected after brief trypsinization and prepared as above. The bottom panels show morphology of cells viewed by phase contrast microscopy of live cultures. Original magnification is 100× (top) or 40× (bottom); bar represents 20 μm.

(C) In the top panel, the equivalent cell diameter of live cultured AMs from $GM^{-/-}$ mice transduced with a control vector (black) or PU.1-expressing vector (hatched) was determined by morphometry as described in Experimental Procedures. In the bottom panel, to measure the effect of PU.1 on adhesion of cultured AMs derived from $GM^{-/-}$ mice, cells transduced with control (black) or PU.1-expressing (hatched) vectors were seeded in ultra-low adherence plastic dishes. Adherence was quantified 2 hr later by removal of nonadherent cells by washing and enumerating adherent cells visualized by fluorescence microscopy at 10× magnification.

lipids and surfactant protein A (SP-A) in cultured AMs derived from $GM^{-/-}$ in the absence or presence of forced PU.1 expression. $AM_{GM}{}^{+/+}{}_{/MHS}$ demonstrated easily detectable uptake of natural surfactant labeled with rhodamine-conjugated dipalmitoylphosphatidylethanolamine (R-DPPE) as previously demonstrated for primary $AM_{GM}{}^{+/+}$ (Yoshida et al., 2001) (Figure 3A). Although $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ failed to internalize R-DPPE, expression of PU.1 in $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ rescued R-DPPE uptake. To determine the role of PU.1 in surfactant lipid degradation, $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ or $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ were incubated with 1-2-phosphatidyl[N-methyl-^{3}H]choline,1,2-dipalmitoyl ([^{3}H]DPPC) and then evaluated for [^{3}H]DPPC breakdown products. PU.1 partially restored surfactant lipid degradation, albeit not to levels seen in primary $AM_{GM}{}^{+/+}$ (Figure 3B). The role of PU.1 in stimulating catabolism of surfactant protein was evaluated by quantifying uptake and degradation of [^{125}I]-labeled SP-A in these cells. Both uptake and degradation of [^{125}I]-labeled SP-A was significantly reduced in $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ compared to $AM_{GM}{}^{+/+}$ ($p < 0.001$) (Figure 3C). Although the effect of PU.1 on SP-A uptake was mild, it normalized SP-A catabolism; SP-A degradation in $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ was significantly increased compared to $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ ($p < 0.01$). These data demonstrate that PU.1 partially corrects abnormalities of uptake and degradation of surfactant lipid and restores SP-A catabolism in AMs of $GM^{-/-}$ mice.

PU.1 Rescues Phagocytosis and Bacterial Killing in AMs from $GM^{-/-}$ Mice

To quantify the phagocytic capacity of AMs in vivo, FITC-labeled latex beads were administered intratracheally to $GM^{+/+}$ or $GM^{-/-}$ mice, and AMs were recovered 6 hr later and assessed by FACS analysis. $AM_{GM}{}^{+/+}$ demonstrated a 3 log increase in the single peak of fluorescence after bead administration (Figure 4A). In contrast, the majority of AMs from $GM^{-/-}$ mice showed minimal fluorescence enhancement, and only a small population showed a 2 log enhancement. Similar evaluation of cultured $AM_{GM}{}^{-/-}$ in vitro showed only a small increase in mean fluorescence after bead administration (Figure 4A). Expression of PU.1 in $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ stimulated a 2 log increase in mean fluorescence of all cells after bead administration compared to cells in the absence of beads. Phagocytosis of specific pathogens and latex beads in cultured AMs was also evaluated by fluorescence microscopy. $AM_{GM}{}^{+/+}{}_{/MHS}$ avidly phagocytosed fluorescently labeled gram-negative and gram-positive bacteria (*E. coli*, *S. aureus*, respectively), yeast particles (zymosan), and latex beads (Figure 4B). In contrast, cultured $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ demonstrated little phagocytic uptake of either bacteria or zymosan and markedly reduced uptake of fluorescent beads. Forced PU.1 expression rescued specific and nonspecific phagocytosis in $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$.

Bacteriocidal activity was assessed in AMs expressing or lacking PU.1 by incubating cultured AMs with live *E. coli* or group B *Streptococcus*. Because of differences in phagocytic capacity, the data were normalized for the phagocytic index of $AM_{GM}{}^{-/-}{}_{/MHS}$, $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$, $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ for each organism. The phagocytic index for live *E. coli* and group B *Streptococcus* in these cell lines (see Experimental Procedures) was 181, 1, 62 and 70, 3, 44, respectively, consistent with the relative levels

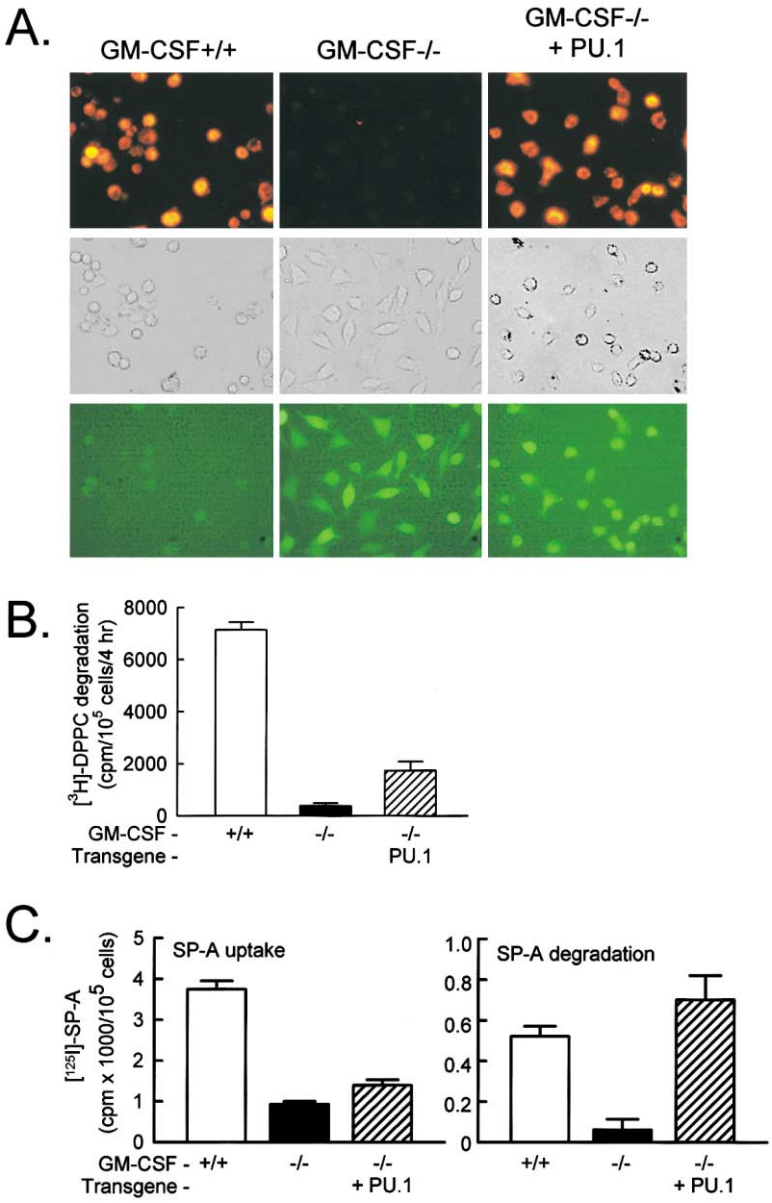

Figure 3. Defective Surfactant Catabolism in AMs of $GM^{-/-}$ Mice and Rescue by Retrovirus-Mediated PU.1 Expression

(A) To evaluate surfactant lipid uptake, cultured AMs (as described in the legend to Figure 1) were incubated with natural surfactant fluorescently labeled with rhodamine-DPPE for 1 hr. Cells were then washed and visualized by fluorescence microscopy using a rhodamine filter to detect R-DPPE (top), by phase microscopy to visualize cells (middle), or with a FITC filter to detect expression of the vector marker gene, GFP (bottom). Original magnification is 20×.

(B) To evaluate surfactant lipid degradation, primary wild-type AMs (open bar) or cultured AMs transduced with a control vector (black bar) or PU.1-expressing vector (hatched bar) were incubated with natural surfactant suspension containing 100 μg/ml [^{3}H]DPPC as described in the Experimental Procedures. The degradation of [^{3}H]DPPC by AMs was estimated by measuring the generation of radioactive products partitioning in the water/methanol phase during extraction from both supernate and cells.

(C) To evaluate SP-A uptake, cultured AMs (as above) were incubated with [^{125}I]SP-A (1 μg/ml, 37°C, 4 hr). Total uptake was calculated from the sum of degradation products in the media and cell-associated radioactivity. Degradation products were calculated as the amount of TCA-soluble radioactivity in the media.

of phagocytic uptake demonstrated for these cells by fluorescence microscopy (Figure 4A). As a positive control, $AM_{GM}^{+/+}{}_{/MHS}$ demonstrated strong killing activity against both organisms (Figure 5). In contrast, $AM_{GM}^{-/-}{}_{/GFP}^{+}$ had significantly less killing capacity for both organisms. PU.1 restored the killing capacity for both organisms, as demonstrated in $AM_{GM}^{-/-}{}_{/PU.1}^{+}$. These data show that expression of PU.1 significantly stimulated pathogen killing independently of its effects on phagocytic uptake of the two organisms.

GM-CSF and PU.1 Rescue Cell-Surface Receptor Expression in $AM_{GM}^{-/-}$

AMs express multiple cell-surface receptors that are involved in the phagocytic internalization of microorganisms (e.g., the mannose receptor [MR]) (Chroneos and Shepherd, 1995) or in proinflammatory cytokine signaling responses to components of the gram-negative (e.g., LPS) and gram-positive (e.g., peptidoglycan) cell walls (e.g., Toll-like Receptor [TLR] 4 and TLR2, respectively) (Aderem and Ulevitch, 2000; Wang et al., 2000). To assess the role of GM-CSF in regulation of these pathways, we used reverse transcription and polymerase chain reaction (RT-PCR) amplification to quantify expression of mRNA for MR, TLR2, and TLR4 (Figure 6). mRNA for MR, TLR2, and TLR4 were readily detectable in $AM_{GM}^{+/+}$ (Figure 6A). In contrast, MR mRNA was undetectable and TLR2 and TLR4 mRNA levels were markedly reduced in primary $AM_{GM}^{-/-}$. Expression of GM-CSF in the lungs of SPC-GM/$GM^{-/-}$ mice restored mRNA levels for all three receptors in $AM_{SPC\text{-}GM/GM}^{-/-}$. Similar to the data for primary AM, mRNA for MR was absent and mRNA for TLR2 and

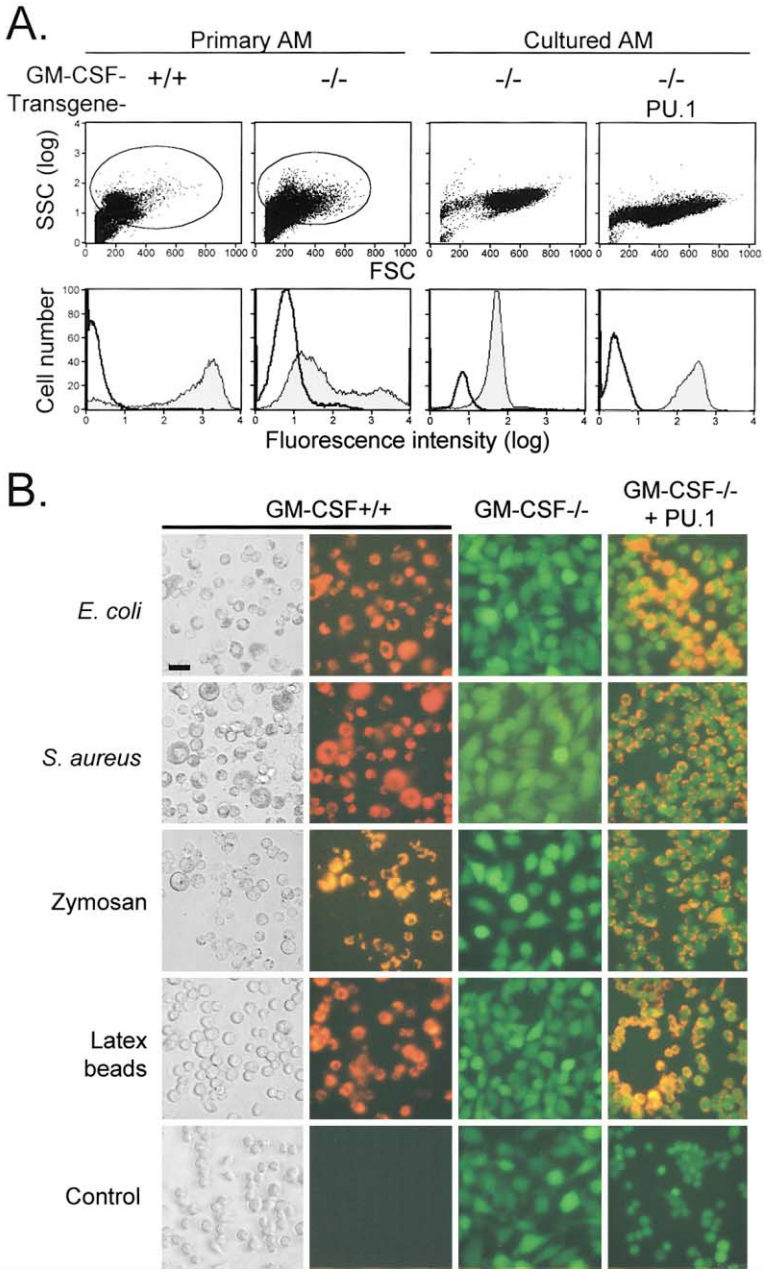

Figure 4. Defective Phagocytosis in AMs of $GM^{-/-}$ Mice and Rescue by Expression of PU.1

(A) FACS analysis of AMs before and after exposure to fluorescent latex beads in vivo and in vitro. Left panels: to assess the effect of GM-CSF on phagocytosis by AMs in vivo, FITC-labeled latex beads were administered to the lungs of $GM^{+/+}$ or $GM^{-/-}$ mice, and primary AMs were recovered after 6 hr and evaluated by flow cytometry. Right panels: to assess the effect of PU.1 on phagocytosis by AMs of $GM^{-/-}$ mice in vitro, cultured AMs transduced with a control vector (−/−) or a PU.1-expressing vector (−/− PU.1), were exposed to FITC-labeled latex beads and evaluated by flow cytometry. Dot plots show forward scatter versus side scatter and the gate used for primary AMs, and histograms show FITC fluorescence versus cell number (shaded curve). Each histogram includes a curve on the left (black line) which shows background autofluorescence of the cells in the absence of beads.

(B) Assessment of phagocytosis in cultured AMs by fluorescence microscopy. Cultured AMs from $GM^{+/+}$ mice (left panels) or from $GM^{-/-}$ mice transduced with either a control (middle panels) or a PU.1-expressing vector (right panels) were evaluated for phagocytic uptake of various pathogens or particles (red-orange color) as indicated. Phase contrast views of $GM^{+/+}$ cells are included for cell visualization; retroviral-transduced AMs from $GM^{-/-}$ mice were visualized by green fluorescence of the GFP marker expressed from control or PU.1 vectors. Original magnification is 40×; bar represents 20 μm.

TLR4 was reduced in cultured $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ (Figure 6A). Importantly, expression of PU.1 in $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ rescued expression of all three receptors.

PU.1 Rescues Toll-Like Receptor Signaling in AMs from $GM^{-/-}$ Mice

Gram-negative bacteria stimulate secretion of proinflammatory cytokines from macrophages through interaction of lipopolysaccharide (LPS) with LPS binding protein, which then signals through a cellular pathway involving CD14, TLR4, and MyD88, ultimately increasing expression of NFκB-regulated genes such as TNFα and IL-6 (Aderem and Ulevitch, 2000). Since $GM^{-/-}$ mice have reduced susceptibility to LPS-induced shock as well as abnormalities of secretion of several NFκB-regulated cytokines (Basu et al., 1997), we evaluated the effect of LPS on cytokine release by $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ and $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ (Figure 6B). LPS failed to stimulate TNFα release from $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ at any dose and only stimulated very low level IL-6 release at high LPS doses. In marked contrast, expression of PU.1 in $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ rescued dose-dependent, LPS-stimulated TNFα and IL-6 secretion that was significantly higher than $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ at all doses tested ($p < 0.001$). Since TLR2 is involved in stimulation of proinflammatory cytokine signaling by gram-positive bacteria (Underhill et al., 1999), we also evaluated the effect of peptidoglycan on cytokine release. Peptidoglycan stimulated dose-dependent TNFα release by $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ (Figure 6B). However, expression of PU.1 in $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ signifi-

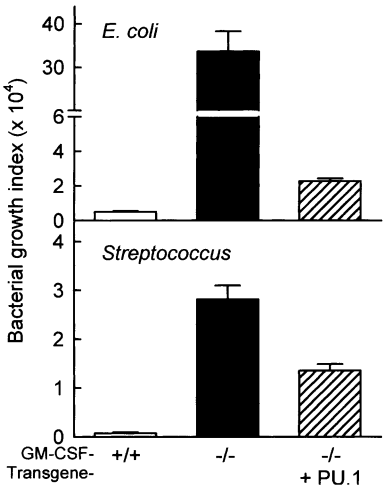

Figure 5. Defective Intracellular Killing of Bacteria in AMs of $GM^{-/-}$ Mice and Rescue by PU.1 Expression

Intracellular killing of live gram-negative (*E. coli*) and gram-positive (*Streptococcus*) bacteria was assessed in cultured AMs from $GM^{+/+}$ mice (open bar) or AMs from $GM^{-/-}$ mice transduced with either a control vector (black bar) or PU.1-expressing vector (hatched bar) as indicated and described in Experimental Procedures. Data are normalized for differences in phagocytic uptake of bacteria. The height of each bar reflects the ability of bacteria to grow within AMs (i.e., inversely related to AM killing of bacteria). This experiment was repeated five times with similar results.

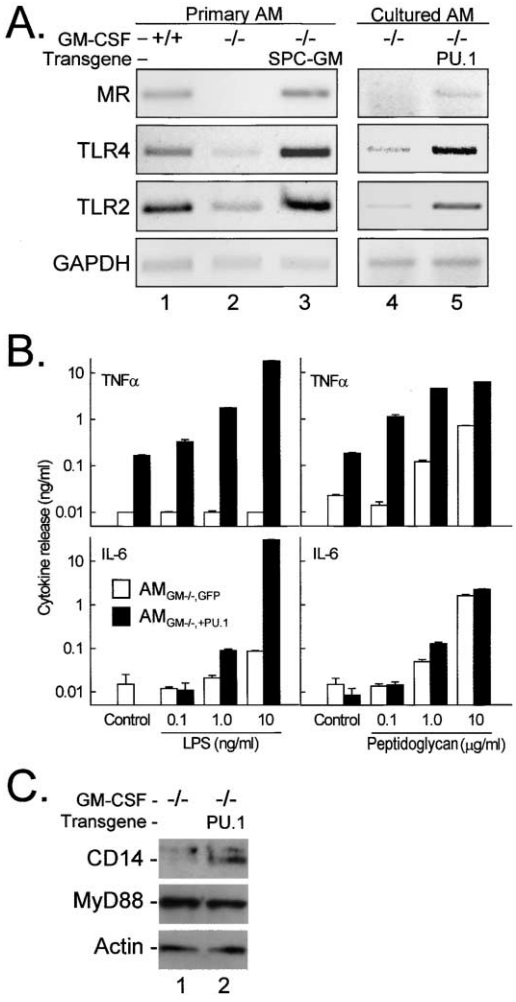

Figure 6. Reduced Cell-Surface Receptor Expression and Inflammatory Cytokine Signaling in AMs from $GM^{-/-}$ Mice and Rescue by Expression of GM-CSF in the Lung In Vivo or Retrovirus-Mediated PU.1 Expression In Vitro

(A) RT-PCR analysis of receptor mRNA in primary AMs and cultured AMs transduced with control or PU.1-expressing vectors. Total RNA was purified from primary or cultured AMs and evaluated by RT-PCR analysis using gene-specific oligonucleotide primers for macrophage mannose receptor (MR), Toll-like receptor (TLR) 4, TLR2, and, as a control, GAPDH.

(B) Assessment of proinflammatory cytokine release by cultured AMs by ELISA. Cultured AMs transduced with control vector (open bars) or PU.1-expressing vector (black bars) were exposed to LPS or peptidoglycan at the indicated concentrations for 16 hr, and supernates were collected and assessed for the presence of TNFα or IL-6 as indicated.

(C) Western blot assessment of expression of components of the TLR4 LPS signaling pathway in AM. Cultured AMs from $GM^{-/-}$ mice transduced with control vector or PU.1-expressing vector were evaluated by Western blotting using anti-murine CD14, anti-murine MyD88, or anti-murine actin antibodies.

cantly increased dose-dependent TNFα release at all doses tested compared to $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ ($p < 0.001$). Similar results were obtained for peptidoglycan-stimulated IL-6 release, although the differences in the absence or presence of PU.1 were less.

Because CD14 expression has been reported to increase as cultured monocytes are induced to differentiate into macrophages and CD14 is important in TLR signaling pathways, we assessed the effect of PU.1 in regulating CD14 and MyD88 levels in AMs by Western analysis. CD14 was markedly reduced in $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ compared to $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$. In contrast, MyD88 did not differ in these cells (Figure 6C). Taken together, these data are consistent with a mechanism by which PU.1 mediates GM-CSF-dependent, pathogen-induced proinflammatory cytokine signaling in AMs via Toll-like receptor pathways.

Discussion

The present studies show that AMs of $GM^{-/-}$ mice have an abnormal phenotype consistent with incomplete differentiation and that levels of the transcription factor PU.1 in AMs correlate precisely with the presence and level of GM-CSF in the lung. Constitutive PU.1 expression in AMs of $GM^{-/-}$ mice in vitro rescued all of the phenotypic abnormalities. The effects of GM-CSF and PU.1 on AM differentiation cannot be attributed to stimulation of differentiation by M-CSF because M-CSF levels were normally low in the lungs of $GM^{+/+}$ mice, but were significantly elevated in the lungs of $GM^{-/-}$ mice and were normalized in SPC-GM/$GM^{-/-}$ mice. These data strongly support the conclusion that GM-CSF is a critical regulator of AM terminal differentiation in the lung and implicate PU.1 as a principal mediator of this effect. This conclusion is supported by the prior observation that PU.1 gene expression can be stimulated by GM-CSF in both murine (Chen et al., 1995) and human (Voso et al.,

1994) hematopoietic progenitors. Further, GM-CSF is known to stimulate survival, proliferation, and differentiation of hematologic progenitors as well as AMs (Akagawa et al., 1988; Chen et al., 1988), and both GM-CSF and PU.1 stimulate expression of large and overlapping sets of genes in mononuclear phagocytes (Hashimoto et al., 1999; Tenen et al., 1997). Recently, lineage-specific development of hematopoietic progenitors was shown to be regulated by the level of PU.1 expression, with high levels directing macrophage differentiation and low levels directing B cell differentiation (DeKoter and Singh, 2000). This finding, together with our results, suggests a potential explanation for the pulmonary lymphocytosis observed in $GM^{-/-}$ mice (Dranoff et al., 1994). While these studies demonstrate that GM-CSF regulates PU.1 levels in AMs, because GM-CSF receptor signaling involves multiple distinct pathways (Sato et al., 1993) further studies will be required to delineate the precise regulatory pathway(s). Also, because most but not all functions of AMs were restored completely by PU.1, our data do not rule out contributions from other transcription factors which have been implicated in late myeloid differentiation (Tenen et al., 1997).

Several lines of evidence support the conclusion that local rather than systemic GM-CSF expression is critical in the regulation of AM differentiation and function in the lung. First, PAP and AM abnormalities in $GM^{-/-}$ mice are corrected by expression of GM-CSF exclusively in the lung and by pulmonary but not intravenous administration of GM-CSF (Huffman et al., 1996; Reed et al., 1999). Second, GM-CSF appears to be tightly compartmentalized between lung and blood because GM-CSF reaches extraordinary levels in the lungs of SPC-GM/$GM^{-/-}$ mice ($\sim$700 pg/ml BAL), yet is undetectable in the serum of these mice (Huffman Reed et al., 1997). Third, the activity of GM-CSF is also compartmentalized between blood and lung: SPC-GM/$GM^{-/-}$ mice have greatly increased AM numbers due to markedly elevated GM-CSF levels in the lungs, while blood leukocyte levels are normal (Huffman et al., 1996). The latter finding would not be expected if blood levels of GM-CSF were elevated (Donahue et al., 1986).

The present study shows that the critical effect of GM-CSF on AMs in vivo is stimulation of their terminal differentiation. We propose a model in which monocytes entering the lung are exposed to levels of GM-CSF stimulating increased PU.1 levels, inducing their further differentiation (Figure 7). This model is supported by several findings. First, multiple, diverse abnormalities in unrelated macrophage functions were demonstrated in AMs of $GM^{-/-}$ mice, including defects in surfactant lipid and protein catabolism, cell morphology, cell adhesion, expression of pathogen pattern recognition receptors, specific and nonspecific phagocytosis, bacterial killing, and Toll-like receptor signaling. Second, all of these abnormalities could be rescued by forced expression of a single "master" transcription factor, PU.1. Third, extensive data demonstrate that PU.1 plays a predominant role in the terminal differentiation of macrophages (Anderson et al., 1998a, 1999; Olson et al., 1995; Scott et al., 1994). Fourth, despite relevance to AM maturation, GM-CSF likely plays a noncritical role in early myelopoiesis and early AM development, because $GM^{-/-}$ and GM R$\beta c^{-/-}$ mice lack hematologic abnormalities in bone

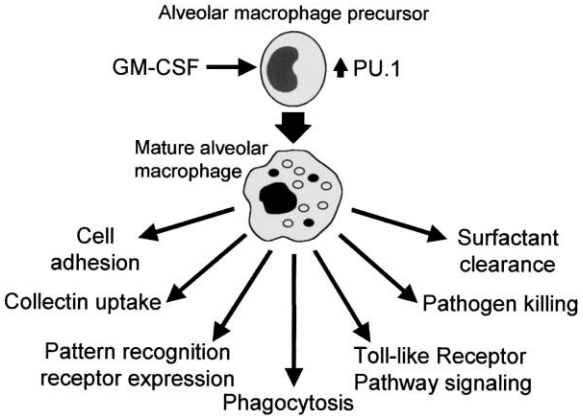

Figure 7. Proposed Role of GM-CSF and PU.1 in Regulation of Differentiation and Innate Immune Function of the Alveolar Macrophage

GM-CSF interacts with macrophage precursors in the lung, stimulating the "master" myeloid transcription factor PU.1. PU.1 is required for terminal differentiation/maturation of AMs, resulting in stimulation of multiple, diverse, and complex functions that enable mature AMs to play critical roles in maintenance of lung homeostasis and innate immune host defense.

marrow and blood and AM numbers are normal (Dranoff et al., 1994; Nishinakamura et al., 1996a; Robb et al., 1995). Consistent with this is the finding that PU.1 is not required for early myeloid gene expression or commitment to the myeloid lineage (Henkel et al., 1999; McKercher et al., 1999; Olson et al., 1995). The observation that PU.1 alters cell adhesion is supported by the finding that both GM-CSF and PU.1 are known to regulate expression of integrins (Feng et al., 1999; Pahl et al., 1993), which are important in cell adhesion. Further, adherence of monocytes to plastic results in increased expression of PU.1, integrin $\beta 2$ mRNA, and hck (hematopoietic cell kinase) (de Fougerolles et al., 2000). Hck is a Src family protein tyrosine kinase expressed in myeloid cells that appears to act as a molecular switch between cell motility and adhesion/spreading in HL60 cells (Chiaradonna et al., 1999). Our observation that brief adherence of AMs to plastic dishes resulted in low level PU.1 expression in $AM_{GM}{}^{-/-}$ (Figure 1B) is consistent with these reports. Because PU.1 positively autoregulates its own transcription (Chen et al., 1995), adherence may also be further stimulated after initiation by a mechanism involving autoregulation of PU.1 expression. The demonstration in AMs of $GM^{-/-}$ mice that PU.1 stimulates uptake and catabolism of both surfactant lipids and proteins in vitro was an unexpected and novel finding of this report. This is supported by the observations that expression of GM-CSF in the lungs of $GM^{-/-}$ mice corrects the abnormalities in surfactant catabolism by AMs (e.g., in SPC-GM/$GM^{-/-}$ mice) and our observation that GM-CSF regulates PU.1 expression in AMs in vivo. This is also supported by the observation that adherence of normal AMs to plastic results in a 5-fold increase in SP-A degradation (Bates and Fisher, 1996). Thus, our data suggest a molecular mechanism for this observation involving an adherence-stimulated increase in PU.1 levels and subsequent effects of PU.1-mediated stimulation of surfactant degradation pathways.

An important finding of the present study is the demonstration that PU.1 critically regulates multiple innate immune functions of AMs. This is supported by the observation that GM-CSF expression in the lungs of $GM^{-/-}$ mice (e.g., SPC-GM/$GM^{-/-}$ mice) rescues these functions in vivo (LeVine et al., 1999) and our demonstration that GM-CSF regulates PU.1 expression in AMs in vivo. Further, recombinant GM-CSF stimulates phagocytosis and bacterial killing by macrophages in vitro and in vivo (Coleman et al., 1988). The MR is a pattern recognition receptor involved in bacterial phagocytosis whose expression is dependent on PU.1 (Eichbaum et al., 1997). Thus, the observation that MR mRNA is absent in $AM_{GM}{}^{-/-}$ and $AM_{GM}{}^{-/-}{}_{/GFP}{}^{+}$ and restored in $AM_{SPC\text{-}GM/GM}{}^{-/-}$ and $AM_{GM}{}^{-/-}{}_{/PU.1}{}^{+}$ provides a potential molecular mechanism to explain these findings. However, the global and parallel regulation of phagocytosis of gram-negative and gram-positive bacteria, zymosan, and latex beads suggests involvement of a more general mechanism. Interestingly, macrophages from hck gene-targeted mice show decreased phagocytosis but have normal hematopoiesis (Lowell et al., 1994), suggesting that hck may be an important link in GM-CSF-mediated regulation of phagocytosis downstream of PU.1. $gp91^{phox}$ and $p47^{phox}$, components of the superoxide-forming phagocyte NADPH oxidase complex important in bacterial killing, are known to be regulated by PU.1 and thus may provide a link in GM-CSF-stimulation of microbicidal activity (Weiser et al., 1987) downstream of PU.1. These data are consistent with the decrease in superoxide production by $AM_{GM}{}^{-/-}$ compared to wild-type AM and the rescue of this abnormality by expression of GM-CSF in the lung (LeVine et al., 1999). The concept that GM-CSF regulates TLR4 mRNA levels in AMs through PU.1 is supported by the observation that PU.1 regulates TLR4 promoter (Rehli et al., 2000). The observation that PU.1 also regulates TLR2 is a novel finding of the present study. Stimulation of TNFα release from macrophages by gram-negative bacteria is mediated by a pathway involving LPS binding protein, CD-14, TLR4, and MyD88, which ultimately results in NFκB and Jun/Fos activation and increased transcription of multiple immune response genes (reviewed in Aderem and Ulevitch, 2000). Thus, our data suggest that PU.1 enables proinflammatory cytokine signaling by stimulating expression of one or more components of this signaling pathway. While regulation of MyD88 does not appear to be involved, further studies will be needed to identify the potentially rate-limiting factors. Taken together, these results demonstrate that GM-CSF is a critical regulator of lung homeostasis and innate immunity in the lung and that its effects are mediated by coordinate regulation of AM gene expression by PU.1.

Experimental Procedures

Mice

$GM^{-/-}$ and SPC-GM/$GM^{-/-}$ mice were generated previously (Dranoff et al., 1994; Huffman et al., 1996) and were maintained in the C57BL/6 background. C57BL/6 mice ($GM^{+/+}$) were from Jackson Laboratories (Bar Harbor, ME). All mice were housed in a pathogen-free barrier facility and studied under procedures approved by the Institutional Animal Care and Use Committee.

Isolation, Culture, and Retroviral Transduction of AMs

Primary AMs were obtained by bronchoalveolar lavage (BAL) as previously described (LeVine et al., 1999). Surfactant lipids and proteins were removed from the cells by sedimentation through PBS containing 15% bovine serum albumin (BSA), 0.5 mM EDTA (450 $\times$ g, 5 min, 4°C) (Sandron et al., 1986). Viability of AMs obtained in this way was greater than 95% for cells of $GM^{+/+}$ and SPC-GM/$GM^{-/-}$ mice as determined by trypan blue staining. Viability of AMs from $GM^{-/-}$ mice was slightly less and was more difficult to evaluate due to the presence of large amounts of surfactant. Brief (1 hr) adherence of AMs to plastic minimized this problem. MH-S is an AM cell line with morphologic and functional features of normal mature AMs, previously derived from normal mice by SV40 transformation (Mbawuike and Herscowitz, 1989). mAM is an AM cell line previously derived from GM-$CSF^{-/-}$ mice by culture in mouse L-929 cell-conditioned media without viral transformation and will be described elsewhere (Z.C.C., unpublished data). Constitutive expression of murine PU.1 was achieved in AMs from $GM^{-/-}$ mice by transduction of mAM cells with a retroviral vector expressing murine PU.1 and, as a selectable marker, also green fluorescent protein (GFP) (DeKoter and Singh, 2000). As a transduction control, mAM cells expressing only the GFP marker were also prepared. Cultured AMs were maintained as previously described for cultured RAW264.7 macrophages (Zsengeller et al., 2000).

Western Blot Analysis

Western analysis was performed as previously described (Anderson et al., 1998b) with primary AMs pooled from three to four mice per group or cultured AMs grown on 100 mm plastic dishes. Primary antibodies included rabbit anti-murine PU.1 (Santa Cruz, CA) (used at 1:400), goat anti-murine actin (used at 1:200), goat anti-murine MyD88 (used at 1:200), or rabbit anti-murine CD14 (used at 1:1600) (all from Santa Cruz). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ) and exposed to Kodak X-Omat AR film.

Morphological and Morphometric Analysis of AMs

Primary AMs collected by BAL without albumin purification or cultured AMs collected by brief trypsinization and washing were sedimented by cytocentrifugation and evaluated by light microscopy as previously described (Dranoff et al., 1994). Digitally captured fluorescence photomicrographs were assessed morphometrically to determine the AM equivalent cell diameter using Metamorph software (Universal Imaging Corp., West Chester, PA) on an IBM-compatible computer.

AM Adherence Assay

To assess cell adhesion, cultured AMs (10^6 cells) were seeded in 35 mm standard plastic or ultra low adherence culture dishes (Costar, Corning, NY). After 2 hr, nonadherent cells were removed by aspiration of the media and gently washing twice with PBS. Adherence was measured by counting the number of AMs per 10$\times$ power field in 5 consecutive, randomly selected fields per dish, using six dishes per AM line.

Uptake and Degradation of Surfactant Lipid and SP-A by AM

The ability of AMs to take up surfactant lipids was assessed using natural surfactant, isolated as described (Ikegami et al., 1996), and mixed with fluorescently labeled R-DPPE (Avanti Polar Lipids, Alabaster, AL) as previously described (Yoshida et al., 2001). Degradation of surfactant lipids by AMs was assessed using natural surfactant (100 μg phospholipid/ml suspension) containing 10 μCi/ml [^{3}H]DPPC (Amersham, Arlington Heights, IL) as previously described (Yoshida et al., 2001). The ability of cultured AMs to internalize and degrade surfactant protein was evaluated using [^{125}I]-labeled SP-A as previously described (Yoshida et al., 2001).

Phagocytosis Assay

Heat-killed *S. aureus*, *E. coli*, or zymosan, as well as 0.1 μm latex beads, each fluorescently labeled with Texas red (Molecular Probes, Eugene, OR), were used to evaluate phagocytosis in cultured AM. Cells were seeded at 10^6/35 mm dish and incubated with bacteria or zymosan at a multiplicity of 100/cell or with latex beads at 10^5/cell

for 2 hr at 37°C. Noninternalized particles were then removed by vigorous and extensive washing with PBS, and cells were fixed in PBS containing 3% paraformaldehyde at 25°C for 15 min and evaluated by fluorescence microscopy as previously described (Zsengeller et al., 2000). To quantify phagocytic uptake in cultured AM, cells were incubated with FITC-labeled 0.1 μm latex beads (Molecular Probes) and washed as above. Cells were then detached by brief trypsinization and evaluated by flow cytometry on a FACSCAN flow cytometer (Becton Dickinson, Franklin Lakes, NJ). All experiments were done in duplicate.

AM Killing of Live Bacteria

Bacterial uptake and killing by cultured AMs was assessed as previously described (Gjomarkaj et al., 1999) using *E. coli* (DH5α; Life Technologies, Gaithersburg, MD) or group B *Streptococcus* (obtained as a clinical isolate from a newborn infant with systemic infection [LeVine et al., 1999]). Bacteriocidal activity was calculated from the number of live bacteria within AMs normalized for the phagocytic index of each and expressed as a bacterial growth index according to the following formula: growth index (GI) = {(bacterial colonies/ml AM cell lysate)/phagocytic index} × 100. The phagocytic index (PI) was determined by assessing 200 consecutive AMs under 80× magnification for the presence and number of bacteria using the following formula: PI = (number of AM) × (% of AMs with bacteria) × (mean number of bacteria/AM).

TNFα and IL-6 Release Assays

Cultured AMs seeded at 2×10^5/well in 24-well plates (Costar) in DMEM containing 10% FBS were exposed to either LPS (*S. typhirium*; Sigma, Saint Louis, MO) at 0.1, 1.0, and 10 ng/ml, or peptidoglycan (Fluka, Milwaukee, WI) at 0.1, 1.0, and 10 μg/ml for 16 hr. AM cell supernatants were then collected, cleared of debris by centrifugation, and TNFα or IL-6 levels were quantified using murine Quantikine kits (R & D, Minneapolis, MN) as directed by the manufacturer. All assays were done in triplicate.

Isolation of RNA and RT-PCR Analysis

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed as previously described (Otake et al., 1998) using the following primer sets: glyceraldehyde 3 phosphate dehydrogenase (GAPDH; 5′-ATTCTACCCACGGCAAGTTCAATGG-3′; 5′-AGGGGCGGAGATGATGACCC -3′), TLR2 (5′-CCTTTGTTTCCTACAGTGAGCAGG-3′; 5′-TCCTGCTGGCCTTCATCCAAGG-3′), TLR-4 (5′-TACTCGAGTCAGAATGAGGACTGG-3′; 5′-TTCGAGGCTTTTCCATCCAATAGG-3′), macrophage mannose receptor (5′-CGGAAGCTGAATCCCAGAAATTCC-3′; 5′-GAATGGAAATGCACAGACGGCTCC-3′).

Statistical Analyses

Numerical data are presented as mean ± SEM. Statistical comparisons were performed using Student's t test with Sigma Plot 4.0 software on an IBM-compatible microcomputer. Statistical significance was taken at $p < 0.05$.

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