Innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR-4

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Bozinovski, Steven, Jessica Jones, Sarah-Jane Beavitt, Andrew D. Cook, John A. Hamilton, and Gary P. Anderson. Innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR-4. Am J Physiol Lung Cell Mol Physiol 286: L877–L885, 2004. First published November 14, 2003; 10.1152/ajplung.00275.2003.—The innate immune inflammatory response to lipopolysaccharide (LPS, an endotoxin) is essential for lung host defense against infection by gram-negative bacteria but is also implicated in the pathogenesis of some lung diseases. Studies on genetically altered mice implicate granulocyte-macrophage colony-stimulating factor (GM-CSF) in lung responses to LPS; however, the physiological effects of GM-CSF neutralization are poorly characterized. We performed detailed kinetic and dose-response analyses of the lung inflammatory response to LPS in the presence of the specific GM-CSF-neutralizing antibody 22E9. LPS instilled into the lungs of BALB/c mice induced a dose-dependent inflammatory response of intense neutrophilia, macrophage infiltration and proliferation, TNF-α and matrix metalloproteinase release, and macrophage inflammatory protein-2 induction. The neutralization of anti-GM-CSF in a dose-dependent fashion suppressed these inflammatory responses by ≤85% when given before or after LPS or after repeat LPS challenges. Here we report for the first time that the physiological expression of Toll-like receptor-4 in lung is reduced by anti-GM-CSF. We observed that lower Toll-like receptor-4 expression correlated with a similar decline in peak TNF-α levels in response to endotoxin. Consequently, sustained expression of key inflammatory mediators over 24 h was reduced. These data expand the understanding of the contribution of GM-CSF to innate immune responses in lung and suggest that blocking GM-CSF might benefit some lung diseases where LPS has been implicated in etiology.

lipopolysaccharide; neutrophil; monocyte; granulocyte-macrophage colony-stimulating factor; Toll-like receptor-4

The intimate relationship between lipopolysaccharide (LPS) and granulocyte-macrophage colony-stimulating factor (GM-CSF) has been recognized since the discovery of this hematopoietin in the supernatant of LPS-conditioned mouse lungs by Sheridan and Metcalf (48) in the early 1970s. We recently demonstrated that a high dose of neutralizing anti-GM-CSF antibody (22E9) suppresses LPS-induced lung inflammation associated with reduced activation of Akt, activator protein-1 (AP-1), and nuclear factor-κB (NF-κB; Ref. 6). As the biology of GM-CSF strongly suggests, it contributes not only to hematopoiesis but also to inflammatory lung immunopathologies (5–7, 25, 28, 44, 46, 56). The objective of this study was to gain a detailed understanding of the effects of neutralization of GM-CSF on lung inflammation triggered by topical exposure of the mouse lung mucosa to LPS in vivo.

LPS is an endotoxin from the outer membrane of grammegative organisms and a primary trigger of innate immunity and acute inflammation, which are essential for successful antimicrobial defense reactions against such organisms (55). In many cell types, LPS induces inflammation by binding to soluble LPS-binding protein, which then facilitates binding to membrane-associated accessory proteins, particularly membrane CD14 (mCD14) and MD-2 and at least one signalingcompetent coreceptor where the mammalian Toll-like receptor-4 (TLR-4) has been implicated (43). Notably, diminished TLR-4 expression on isolated alveolar macrophages from GM-CSF-deficient mice can be rescued by forced expression of the GM-CSF-regulated transcription factor PU.1, which restores priming and endotoxin responses (49). LPS triggers activation of myeloid differentiation protein (MyD88), MyD88-associated protein Mal, TNF-receptor-associated factor-6 (TRAF-6), IL-1-receptor-associated kinase (IRAK), NFκB, and Akt (6, 9, 16, 34). These signal transduction intermediary molecules in turn upregulate inflammatory mediator and chemokine synthesis and also trigger preformed mediator release. In particular, LPS is an important inducer of the release of the proinflammatory mediator TNF-α, proteases, and chemokines. In lungs, LPS is particularly effective in triggering alveolar macrophages and respiratory epithelium.

Inhaled LPS is also an important cause of environmentally induced airway disease in occupations where exposure to bacteria-contaminated organic dusts (bioaerosols) is common (23). In human volunteers and in several animal species, lung aerosol challenge or LPS instillation causes a neutrophil-rich inflammatory response (5, 27, 31, 46, 47). LPS is released from pathogens such as Moxarella catarrhalis, Haemophilus influenzae, and Pseudomonas aeruginosa, which are known to acutely infect and also to colonize the lungs of patients suffering from asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (15, 17, 22, 26, 30). LPS from these organisms has therefore been implicated in worsening airway inflammation and nonviral exacerbations of these conditions (15, 31). Most recently, environmental exposure to LPS-containing bioaerosols was identified as actually causing as well as exacerbating asthma (15, 21, 25, 37, 38). Therefore, understanding the regulation of inflammation triggered by...
delivering LPS into lungs is likely to be relevant to both enhancing host defense in gram-negative lung infections and dampening detrimental inflammation in some chronic lung diseases or their exacerbations.

Because little is known about the effects of anti-GM-CSF antibodies given prophylactically before LPS, and nothing is known of the effects of anti-GM-CSF on post-LPS- and multiple-LPS-challenge responses in lungs, we have performed detailed dose-response and time-course experiments. Our data provide new evidence for the role of GM-CSF in LPS-induced lung inflammation, and, to the extent that LPS is involved in the induction and exacerbation of some lung diseases, our data also suggest the potential therapeutic utility of blocking GM-CSF in vivo.

**METHODS**

**Animals.** Specific-pathogen-free male BALB/c mice (IA2, body wt, 20–24 g; 6–7 mo of age) were obtained from the Biological Research Facility (Perth, Australia). Animals were house-housed at 20°C on a 12:12-h day-night cycle and were fed a standard diet of Purina mouse chow with water allowed ad libitum. All procedures were approved by the Animal Experimentation Ethics Committee of the University of Melbourne and conformed to international standards of animal welfare as specified in the guidelines of the National Health and Medical Research Committee of Australia. In particular, animals were carefully observed for signs of respiratory distress or discomfort after LPS instillation during initial dose-ranging studies.

**Induction of inflammation by transnasal instillation of LPS.** Inflammation was induced by instillation of graded doses of LPS (Escherichia coli serotype 026:B6 Sigma) in 35 μl of PBS vehicle into the lungs of groups of 8–10 mice using a transnasal challenge method (5, 51). Mice were anesthetized with 2% enflurane (Abbott) in air. Solution (35 μl) was applied to the nares. Because mice are obligate nose breathers, this method uniformly instilled fluids through all lung regions, which was confirmed in prior pilot experiments with the tracer pigment monastral blue (Sigma). These tracer studies indicated that materials delivered by this route in mice readily accessed lung tissue including cells within the thin airway walls, which is in contrast to fluid instillation in larger rodents and humans. The maximum diffusion distance in intrapulmonary airways, which can be accessed from either the airway epithelium or the immediately adjacent alveoli, is less than ~8 μm (total airway wall width, ~16 μm; epithelium, ~10 μm; submucosa, ~6 μm) and is less than ~2 μm in alveoli. Solutions administered to the mice alone or in combination and at specified time points were as follows: 1) PBS; 2) PBS containing LPS at the indicated dose; 3) PBS containing anti-GM-CSF MAb (22E9, endotoxin-free rat anti-mouse neutralizing IgG2a, purified in house; see below) at the indicated dose; 4) PBS containing isotype control (rat anti-mouse IgG2a MAb of irrelevant specificity); 5) heat-inactivated 22E9 in PBS; 6) the potent anti-inflammatory glucocorticosteroid dexamethasone (tissue-culture grade; Sigma); or 7) its vehicle (PBS with 0.001% vol/vol ethanol).

We previously demonstrated that IgG2a antibodies instilled into the lung via this method do not traffic to the systemic circulation; therefore, all effects observed in this study were due to local neutralization of GM-CSF in the lungs (54). In dose-response experiments, groups of mice received a single LPS challenge at time 0. In one series of experiments performed to determine the effects of anti-GM-CSF on established inflammation, mice received a second LPS challenge (10 μg/mouse) 24 h after the first challenge (10 μg/mouse) at a point when neutrophilia from the first LPS challenge was maximal. In the later series of experiments, treatments were administered 3 h before the second LPS challenge. For necropsy, mice were anesthetized with ketamine-xylazine (15 and 30 mg/kg ip, respectively), and a blood sample was drawn from the vena cava for serum. Inflammatory responses were measured by bronchoalveolar lavage (BAL) of the lungs via tracheotomy (SP30 Duran polyethylene tubing) proximal to the larynx; four aliquots of 0.3 ml of PBS at room temperature were pooled. Fluid recoveries were 85 ± 5% and did not differ significantly between groups. Total cell counts and viabilities were determined using ethidium bromide-acridine orange (Molecular Probes) fluorescent viability stains and were counted with a ZEISS Axioshot microscope and a Neubauer hemocytometer. Cytometric preparations (Shandon Cytospin 3) were prepared using 100 μl of neat BAL fluid that was air dried and stained using Diff-Quick (Dade Behring, Sydney). Cell types and mitotic figures were identified according to standard morphological criteria and were enumerated as the multiple of the total cell count and differential cell type percentage after counting at least 500 cells/slide under oil immersion at ×1,000 magnification (AxioLab, Zeiss).

**Preparation of 22E9 and IgG2a isotype control.** Endotoxin-free 22E9 and its isootype control IgG2a were prepared from ammonium sulphate hybridoma precipitates via affinity chromatography. Final samples were concentrated, dialyzed extensively against endotoxin-free PBS, and lyophilized. Heat-inactivated 22E9 was prepared by boiling sealed aliquots of the antibody for 3 h. Heat-inactivated 22E9 failed to neutralize recombinant murine GM-CSF in the specific GM-CSF FDC-P1 cell proliferation bioassay. Lack of endotoxin contamination was confirmed by bioassay; accordingly, pure 22E9 administered at a dose of 100 μg/mouse, which was the highest dose used in this study, did not induce any detectable neutrophilic response in mice in vivo. The 22E9 has very high specificity for GM-CSF, and in the context of this study, 22E9 has no measurable interaction with neutrophil hematopoietins [e.g., granulocyte CSF (G-CSF), IL-3, stem cell factor (SCF), and -CSF-1], inflammatory mediators [e.g., TNF-α, chemokines [e.g., growth-related oncogene (GRO) and macrophage inflammatory proteins (MIPs)], or adhesion molecules required for inflammatory cell diapedesis, at concentrations up to 10 mg/ml.

**ELISA studies.** Murine TNF-α and GM-CSF levels were determined by standard sandwich ELISA (R&D Biosystems). The limits of detection were 10 pg/ml for TNF-α and 15 pg/ml for GM-CSF.

**GM-CSF bioassay.** Concentrations of GM-CSF in individual BAL samples were calculated by comparison to a GM-CSF standard (Genzyme) curve, starting at 5 U/ml, using proliferation of the GM-CSF-responsive cell line FDC-P1 measured by 3H-thymidine incorporation. The 22E9 (1 μg/ml) completely suppressed proliferation in this assay. Duplicate undiluted BAL fluid samples were incubated overnight for 12 h with FDC-P1 (10 cells/well) at 37°C in 5% CO2. Cells were then pulsed with 3H-thymidine for 16 h, harvested, and counted (beta scintillation counter, Beckman, Irving, CA). After subtraction of background, bioactivity levels were determined by linear interpolation. The specificity of the bioassay for GM-CSF was assessed by blockade of proliferation with 22E9 (1 μg/well) in control wells.

**Staining for fluorescence-activated cell sorting and flow cytometry.** BAL-fluid cell populations were pooled, washed twice in PBS containing 1% FCS, and resuspended on ice at a concentration of 1 × 106 cells/ml. After cells were preblocked with an antibody cocktail directed against Fc receptors (Fc blocker, Pharmingen), cells were incubated for 30 min on ice with optimized amounts of directly conjugated phycoerythrin- or FITC-labeled rat anti-mouse IgG2a, antibodies directed against the surface markers B220, CD3, CD4, CD8a, CD25, and CD44 (Pharmingen). Directly conjugated rat anti-mouse IgG2a, antibodies of irrelevant specificity were used as isotype controls. Cells were washed twice on ice, and 10 μg of propidium iodide (Molecular Probes) was added to cell suspensions immediately before acquisition to exclude dead cells and doublets. Data were acquired at 10,000 particle-gated events on a FACScan cytometer, and were analyzed using CellQuest software (Becton Dickinson).

**RNA extraction and quantitative real-time PCR.** Lungs were perfused of blood (via right ventricular perfusion with 10 ml of warmed
LPS induces dose-dependent acute neutrophilic inflammation. Consistent with other lung LPS-exposure models (5, 27, 31, 51, 57), LPS induced a graded, dose-dependent neutrophilia (Fig. 1) that was assessed 24 h after challenge. The neutrophil response threshold was 0.3 μg/mouse, and the response increased progressively up to 100 μg/mouse; however, at doses ≥30 μg of LPS lung hemorrhage occurred, and mice became visibly distressed. We therefore selected the highest tolerated dose, namely, 10 μg/mouse, for subsequent experiments and usually measured outcomes at 24 h, which was the point of the most intense inflammatory response.

Kinetics of LPS-induced inflammatory response. In detailed kinetic studies, the neutrophilia induced by 10 μg of LPS was evident within 2 h and markedly elevated at 6 h; it peaked at 24 h. The neutrophil level decreased markedly by 48 h and was resolved by 72 h (Fig. 2A). In kinetic studies, GM-CSF levels measured by specific ELISA increased sharply at 1 and 2 h post-LPS, decreased dramatically by 6 h, and increased slightly at later times (Fig. 2B). Because ELISA determinations of GM-CSF may not reflect biological activity, we made parallel determinations of GM-CSF bioactivity that were consistent with the kinetic changes monitored by immunoassay (Fig. 2B). However, it should be noted that in vivo evidence exists to show that absolute GM-CSF levels can be dramatically underestimated due to highly effective receptor-mediated removal by neutrophils (36).

Because macrophages have been closely linked to chronic lung inflammation, we measured the kinetics of macrophage increase in the lung. Macrophage numbers initially decreased sharply by 1 h, which presumably reflects acute activation; such a disappearance was observed previously in murine peritoneal cavity inflammation models (3). Macrophage numbers rebounded to prechallenge levels at 6 h then slowly increased to salivary response over 72 h. LPS was instilled at a dose of 10 μg/mouse, and necropsies were performed on animals in groups exactly at the time points indicated. A: BAL-fluid cells were assessed by viability, total cell count, and differential staining. Note the macrophage (macs) “disappearance reaction” between 30 min and 1 h postchallenge. Data are expressed as absolute number of cells per milliliter for 8–12 mice/dose. *P < 0.05 compared with PBS control using ANOVA and Newman-Keuls multiple-comparison test.

Fig. 2. Kinetics of the LPS response over 72 h. LPS was instilled at a dose of 10 μg/mouse, and necropsies were performed on animals in groups exactly at the time points indicated. A: BAL-fluid cells were assessed by viability, total cell count, and differential staining. Note the macrophage (macs) “disappearance reaction” between 30 min and 1 h postchallenge. Data are expressed as absolute number of cells per milliliter for 8–12 mice/dose. *P < 0.05 compared with PBS control. B: kinetics of granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion in BAL fluid as assessed by ELISA in mice challenged with 10 μg/mouse LPS and the corresponding bioactivity measurement at the same time points. Neut, neutrophils; lymph, lymphocytes; 3H-Tdr, [3H]thymidine.
12 h at which time levels remained constant until day 3, when, in contrast with neutrophils, an additional increase was observed. Interestingly, this increase in macrophage numbers at 72 h did not appear to be due solely to recruitment, since we observed a three- to fourfold increase in mitotic figures between 48 and 72 h (Fig. 3), which suggests in situ replication (4). Local macrophage replication is a characteristic of inflammatory lung reactions in animals and in clinical conditions including COPD (4, 13).

There was a small but significant influx of lymphocytes that was barely evident at 2 h and was maximal by 12 h (2–4% of all cells at 24 h post-LPS) that was composed predominantly of CD3+CD8+ and CD3+CD4+ helper T cells (98%; 43:67 CD4/CD8 ratio). Many CD3+ cells (46%) expressed CD44, a memory/activation marker, but all were negative for CD25, a component of the IL-2 receptor complex expressed on proliferating T lymphocytes and regulator T cells. Residual lymphocytes were mostly B220+ B cells (~2%). Epithelial desquamation did not occur, and metachromatic cells (mast cells and basophils) were absent from lavages at all time points. Eosinophils were not observed.

Effects of anti-GM-CSF antibodies. Neutralization of purified anti-GM-CSF antibody (22E9, 1–100 μg/mouse) but not its control isotype (IgG2a) dose-dependently suppressed neutrophilic inflammation by up to 85% when administered directly into the lungs 3 h before LPS challenge (Fig. 4). Importantly, anti-GM-CSF therapy also significantly reduced macrophage replication measured as mitotic figures at 72 h (Fig. 3). Heat inactivation destroyed the inhibitory activity of the neutralizing antibody. Because neutrophilic inflammation in many inflammatory lung diseases is notably refractory to steroid therapy (5, 27, 42), we compared the effects of 22E9 to those of the highly potent anti-inflammatory glucocorticoid dexamethasone (20 μg/mouse instilled transnasally into lungs at a dose equivalent to an inhaled total dose of >8 mg in a 70-kg human). This steroid did not significantly inhibit LPS-induced neutrophilic inflammation and slightly but not significantly augmented the response (Fig. 4A). This dose of steroid induced marked thymic atrophy, which is a positive control for steroid action in vivo, and suppressed expression of TNF-α transcript (data not shown).

We also assessed whether 22E9 was effective when administered after the LPS insult. As seen in Fig. 4B, the neutralizing
anti-GM-CSF antibody markedly suppressed neutrophilic inflammation when administered even as late as 6 h post-LPS challenge. Furthermore, in animals that had received two LPS challenges at 24-h intervals, 22E9 markedly and significantly reversed established airway inflammation (Fig. 4C).

Sustained expression of key inflammatory mediators is dampened by anti-GM-CSF. We determined the effects of anti-GM-CSF therapy on TLR-4, TNF-α, MIP-2, and G-CSF expression over a 24-h time course via quantitative real-time PCR (Fig. 5). Anti-GM-CSF antibody suppressed basal TLR-4 levels by 21% (saline, 98 ± 10 vs. anti-GM-CSF, 77 ± 5%) before LPS challenge. This basal decline was not reflected by significant changes in basal expression of TNF-α, MIP-2, and G-CSF; baseline release of these mediators is most likely biologically negligible as evidenced by the large fold increase in response to endotoxin. The reduction in basal TLR-4 levels was, however, consistent with a similar decline in peak TNF-α expression (34%) 2 h post-LPS challenge (saline, 34.7 ± 1.3-fold vs. anti-GM-CSF, 22.9 ± 1.9-fold). In contrast, peak expressions of MIP-2 and G-CSF at 2 h were not reduced by anti-GM-CSF.

To address these contrasting findings, we next assessed TNF-α protein secretion into the lavage compartment by ELISA (Fig. 6). Release of TNF-α is an early event in response to endotoxin that itself has profound effects on the regulation of inflammatory mediators. Here we observed an initial burst of TNF-α release at 2 h that was unaffected by pretreatment with anti-GM-CSF. We previously reported that this may correspond to preformed TNF-α stores that are rapidly released under inflammatory conditions (6). This anti-GM-CSF-resistant release of TNF-α at 2 h may then drive and maintain expression of mediators such as MIP-2 and G-CSF at this earlier time point. However, anti-GM-CSF does suppress peak TNF-α levels (4 h) by 37% (saline, 5.2 ± 0.2 vs. anti-GM-CSF, 3.2 ± 0.2 ng/ml), and this is very consistent with the decline in peak TNF-α transcription (34%) at 2 h previously mentioned. As a consequence of reduced peak TNF-α secretion and reduced peak NF-κB/AP-1 activation shown previously (6), we observed a decline in the sustained expression of all inflammatory mediators including TNF-α measured at the later 24-h time point.

We also observed induction of matrix metalloproteinase 9 (MMP-9) as assessed by real-time PCR (Fig. 7A). Consistent with the increase in transcript, secreted MMP-9 was detected in the BAL fluid of LPS-challenged mice using gelatin zymography (92 kDa corresponding to MMP-9), which was suppressed by 55% in anti-GM-CSF-treated mice (Fig. 7B). Because latent MMPs are activated in the zymographic procedure, it is not possible to infer net free-protease activity in BAL fluid from these measurements. We therefore assessed net gelatinase activity in pooled lavagates. LPS induced gelatinase activity in pooled BAL fluid, and this was suppressed by anti-GM-CSF (Fig. 7C). These data indicate that GM-CSF...
regulates not only net MMP induction but also net proteolytic activity. Interestingly, we observed a decline in net basal gelatinase activity in anti-GM-CSF-treated mice. The cause of this decline was not associated with MMP-9 expression, which implicates the negative regulator of this protease, tissue inhibitor of MMP-1 (TIMP-1). Previously we reported that neutralizing antibodies have some biological activity associated with a modest increase in Fc-receptor-mediated activation of AP-1 (6). More recently, AP-1 has been shown to bind to the promoter region of TIMP-1, which is a process integral to expression of this inhibitor (20).

**DISCUSSION**

The principal objective of the present study was to characterize in detail the effects of neutralizing GM-CSF in a mouse model of LPS-induced lung inflammation. GM-CSF has proinflammatory properties because of its action on neutrophils and cells of the monocyte macrophage lineage (7, 32) and directly contributes in NF-κB-dependent lung inflammation in part via activation of the upstream kinase Akt/PKB (6). Recombinant GM-CSF augments leukocyte leukotriene and superoxide anion production (50), enhances allergic sensitization (28), and when selectively overexpressed as a transgenic product in the rat lung epithelium, GM-CSF promotes inflammation and fibrosis (60). In our study, we observed that anti-GM-CSF antibody administered either prophylactically before LPS or therapeutically even 6 h after LPS challenge, i.e., the time point when neutrophil number was nearly maximal, effectively reduced neutrophil counts in a dose-dependent manner at 24 h. Moreover, anti-GM-CSF reversed established inflammation and suppressed secondary LPS challenges. Because we observed increased GM-CSF concentrations in BAL, and because GM-CSF inhibits neutrophil apoptosis (41, 58), the ability of anti-GM-CSF antibody to reverse established neutrophil inflammation was probably due in part to apoptosis. This suggestion is reinforced by the higher frequency of nonviable acridine orange-stained cells that we observed in 22E9-treated animals. GM-CSF acts directly as a survival factor for neutrophils and macrophages (11, 24). GM-CSF levels are markedly elevated in lung macrophages and airway epithelium by exposure to LPS in vitro (35), and at least in vitro this induced GM-CSF enhances neutrophil survival (11, 24). Some of the associated decline in BAL-fluid macrophage numbers could be due to a similar apoptotic cell death: we observed GM-CSF-sensitive macrophage replication that is consistent with the ability of GM-CSF to induce lung macrophage replication in vitro (40) and the increase in macrophage numbers observed after targeted GM-CSF overexpression in the lung (60). We did not, however, observe multinucleated macrophages, which were previously observed in GM-CSF-treated alveolar macrophages in vitro (29).

We also found that as in many clinical inflammatory lung diseases where neutrophilic inflammation is prominent (15, 22, 23, 37, 38, 47), the LPS-induced inflammatory reaction was markedly refractory to glucocorticosteroids. We used a high dose of steroid, the activity of which was confirmed by measuring thymic atrophy and repression of TNF-α transcript in vivo. Glucocorticosteroids are known to promote neutrophil survival (11, 24). Some previous studies (but not the majority)
have suggested that high-dose systemically administered glucocorticosteroids ameliorate the effects of LPS administered to the lungs of mice (19, 60). The most likely reason for these apparent discrepancies is that systemically administered glucocorticosteroids exert a strong leukopenic effect and can act directly on bone marrow to suppress myelopoiesis and leucocyte release into the blood. Consistently, numerous effects of LPS are known to be steroid resistant in mice in vivo (2, 27, 33, 39, 45). In accordance with our mouse model, highly potent and selective inhaled glucocorticosteroids have no inhibitory effect on inflammation triggered by inhaled LPS-laden bioaerosols in humans (10).

TNF-α is considered to be a key inflammatory mediator in lung pathology, and its levels are elevated in lung diseases where LPS is implicated and neutrophilic inflammation is prominent. TNF-α is released by activated alveolar macrophages in response to LPS and is a potent chemoattractant for neutrophils. In concert with chemokines, TNF-α also promotes release of reactive oxygen species, elastase, and other proteases including MMPs that are implicated in lung tissue damage. In mice, neutralization of TNF-α was previously shown to blunt instilled LPS-induced lung inflammation (18) although to a lesser degree than that observed in the present study. LPS triggers preformed TNF-α release from mast cells, which is consistent with the very rapid increase in TNF-α levels in our study, and such TNF-α is a potent stimulus for secondary induction of chemokines that in turn promote leucocyte influx and survival. Our data also suggest that in the lung, basal GM-CSF expression controls LPS-induced TNF-α levels. A similar dependency of TNF-α levels has been observed in an endotoxin-induced systemic murine sepsis model (53) and extends to other animal models (7). Previously it was reported that TLR-4 levels in alveolar macrophages isolated from GM-CSF-deficient mice express lower levels of TLR-4 compared with macrophages purified from wild-type mice (49). Here we extend this study and report for the first time that in vivo, expression of TLR-4 in lungs is in part maintained by basal GM-CSF and that neutralizing LPS-induced GM-CSF also blocks peak TLR-4 levels during an inflammatory response. As a consequence of the reduction in TLR-4, we suggest that this has a direct effect on TNF-α expression due to a similar decline in expression of the cytokine over the 24-h challenge.

We previously investigated the molecular pathway(s) that mediates this reinforcement of TNF-α levels in vivo and in particular demonstrated the GM-CSF-dependent activation of the transcription factors AP-1 and NF-κB in response to LPS (6). Other models do suggest that TNF-α levels in diverse inflammatory conditions are dependent on GM-CSF expression (53). Of possible relevance to the neutrophilia observed in our studies is the fact that TNF-α itself has variable effects on neutrophil survival, but it augments GM-CSF-induced survival of neutrophils (56). It is presently unclear whether GM-CSF regulation of TNF-α also contributed to the marked suppression of MMP-9 induction observed in the present study, as both LPS and TNF-α induce MMP-9 in diverse cell systems. These proteinases are released by multiple cell types in the lung, particularly epithelium and macrophages and, among other functions, contribute to inflammatory cell infiltration into inflamed lungs. MMP-9 induction was previously associated with the development of emphysema in lungs exposed to instilled LPS and acute lung injury following systemic LPS exposure (1, 8, 12, 14, 52, 59, 61).

The striking effects of anti-GM-CSF in our model suggest the possible therapeutic utility of blocking GM-CSF in human lung diseases where high neutrophil numbers, protease induction, and TNF-α overproduction are believed to be central agents in disease pathogenesis. Because our model shows some similarities to the neutrophilic inflammatory reactions observed in occupational lung diseases caused by LPS-containing bioaerosols, the present findings may suggest novel approaches to the treatment of this condition. LPS has also been implicated in the etiology of disease induction and exacerbation in asthma, cystic fibrosis, and COPD (15, 22, 23, 37, 38, 47), and it remains possible but unproven that neutralization of GM-CSF might provide some benefit to these conditions.

In summary, the marked suppression of LPS-induced inflammation by anti-GM-CSF antibody that we have described, when administered prophylactically or therapeutically, suggests that GM-CSF is a key mediator of LPS-induced lung inflammation. Because GM-CSF regulates inflammation through TLR-4 expression, anti-GM-CSF strategies might have therapeutic utility in disease conditions where signaling via this receptor is implicated.

GRANTS

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