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Local Delivery of GM-CSF Protects Mice from Lethal Pneumococcal Pneumonia

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The growth factor GM-CSF has an important role in pulmonary surfactant metabolism and the regulation of antibacterial activities of lung sentinel cells. However, the potential of intra-alveolar GM-CSF to augment lung protective immunity against inhaled bacterial pathogens has not been defined in preclinical infection models. We hypothesized that transient overexpression of GM-CSF in the lungs of mice by adenoviral gene transfer (Ad-GM-CSF) would protect mice from subsequent lethal pneumococcal pneumonia. Our data show that intra-alveolar delivery of Ad-GM-CSF led to sustained increased pSTAT5 expression and PU.1 protein expression in alveolar macrophages during a 28-d observation period. Pulmonary Ad-GM-CSF delivery 2–4 wk prior to infection of mice with Streptococcus pneumoniae significantly reduced mortality rates relative to control vector-treated mice. This increased survival was accompanied by increased inducible NO synthase expression, antibacterial activity, and a significant reduction in caspase-3-dependent apoptosis and secondary necrosis of lung sentinel cells. Importantly, therapeutic treatment of mice with rGM-CSF improved lung protective immunity and accelerated bacterial clearance after pneumococcal challenge. We conclude that prophylactic delivery of GM-CSF triggers long-lasting immunostimulatory effects in the lung in vivo and rescues mice from lethal pneumococcal pneumonia by improving antibacterial immunity. These data support use of novel antibiotic-independent immunostimulatory therapies to protect patients against bacterial pneumonias.

S. pneumoniae (the pneumococcus) is the most prevalent pathogen causing community-acquired pneumonia, septic meningitis, and otitis media and is known to frequently progress to invasive pneumococcal disease associated with high morbidity and mortality rates worldwide. The increase in incidence and global dissemination of multidrug-resistant clones of S. pneumoniae necessitates the development of novel lung-active antibiotic substances. It also requires the development of adjunctive antibiotic-independent strategies, including prophylactic, immunostimulatory approaches to improve lung innate immunity against S. pneumoniae infections in both immunocompetent and immunocompromised patients (1–3). Our group has shown that experimental maneuvers leading to increased intra-alveolar accumulation of mononuclear phagocytes significantly increased survival of mice challenged with S. pneumoniae (4, 5).

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However, there are only a few inconclusive reports that have directly addressed the issue of enhanced immunostimulation to improve lung innate immunity against bacterial challenge (6).

GM-CSF is a hematopoietic growth factor and cytokine that exerts pleiotropic effects on survival, proliferation, differentiation, priming, and activation of myeloid and nonmyeloid cells (7). In the lung, GM-CSF is primarily released by epithelial cells and macrophages and regulates both pulmonary surfactant homeostasis and macrophage-mediated protective immunity (8, 9). Binding of GM-CSF to its receptor triggers activation of the JAK/STAT5 signaling pathway and increased gene expression of the myeloid-specific transcription factor PU.1, which in turn mediates terminal differentiation and is necessary for the function of resident alveolar macrophages (8–10). In humans, disruption of the GM-CSF signaling pathway through GM-CSF–neutralizing antibodies or mutations in the GM-CSFR results in the development of pulmonary alveolar proteinosis, which is characterized by progressive accumulation of surfactant lipids and proteins within alveoli and in alveolar macrophages and abnormalities of host defense (8, 11–13). Absence of GM-CSF in gene-targeted mice results in the development of a pulmonary alveolar proteinosis-like disorder similar to the human disease (14, 15). At the same time, deactivation of the GM-CSF in mice increases their susceptibility to pulmonary and systemic infections to a wide range of pathogens, including Gram-negative and -positive bacteria, mycobacteria, fungi, parasites, and viruses (14–24). Previous reports have demonstrated that rGM-CSF delivered to the airways of GM-CSF knockout mice can improve host defense against group B streptococci, whereas aerosolized GM-CSF results in faster clearance of group B streptococci even in wild-type mice (19). Taken together, these data support a critical role for endogenous cytokines such as GM-CSF in the regulation of lung-protective immunity against inhaled pathogens.

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However, whether GM-CSF is able to prophylactically enhance lung-protective immunity against clinically relevant pathogens such as *S. pneumoniae* when prophylactically given into the airways has not been addressed in the literature. Development of adjunctive antibiotic-independent therapies for patients with risk of developing drug-resistant pneumococcal pneumonia would have important clinical applications with improvement of outcomes and reduction of costs.

### Materials and Methods

#### Animals

C57BL/6N mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All animal experiments were approved by our local government authorities.

#### Reagents

Recombinant murine GM-CSF (rmGM-CSF) was purchased from PeproTech (London, U.K.). Rabbit anti-PU.1 polyconal Ab, rabbit anti-phosphorylated STAT5 Ab, rabbit anti-STAT5 Ab, rabbit anti-cleaved caspase-3 Ab, rabbit anti-inducible NO synthase (iNOS) Ab, and rabbit anti-GAPDH Ab were obtained from Cell Signaling Technologies (Danvers, MA). Mouse anti-β-actin Ab was from Sigma-Aldrich (Deisenhofen, Germany), and peroxidase-conjugated donkey anti-rabbit polyclonal IgG (H+L) was from Jackson ImmunoResearch Laboratories (Suffolk, U.K.). All Abs used in the current study for flow cytometry analysis including anti-CD11c PE-Cy5.5, anti-CD11b PE-Cy7, anti-MHC class II (MHC-II) PE, and anti-Ly6G/Ly6C (GR-1) PE-Cy7 were purchased from BD Biosciences (Heidelberg, Germany). Anti-F4/80 FITC and anti-F4/80 allophycocyanin were from Serotec (Düsseldorf, Germany). The MACS kit and CD11c beads for purification of CD11c+ cells from lung tissue were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

#### Culture and quantification of *S. pneumoniae*

For infection experiments, we used the capsular group 19 *S. pneumoniae* strain EF3030, which is characterized by a relatively low virulence in mice (5, 25, 26). The bacteria were grown in Todd–Hewitt broth (Oxoid, Wesel, Germany) supplemented with 20% FCS to mid-log phase, and aliquots were snap frozen in liquid nitrogen and stored at −80°C until use (5, 27, 28). Quantification of pneumococci was done by plating thawed aliquots in 10-fold serial dilutions on sheep blood agar plates (BD Biosciences), followed by incubation of the plates at 37°C/5% CO2 for 18 h. Whole lung washes were centrifuged (1400 rpm, 4°C, 9 min) and cell pellets were pooled and resuspended in RPMI 1640/10% FCS and total CFU in the respective 1.5 and 4.5 ml BAL fluid aliquots collected from *S. pneumoniae*-infected mice were quantified by plating 10-fold serial dilutions on sheep blood agar plates (BD Biosciences), followed by incubation of the plates at 37°C/5% CO2 for 18 h. Whole lungs were diced (1000 rpm, 4°C, 9 min) and cell pellets were pooled and resuspended in RPMI 1640/10% FCS and total numbers of BAL fluid leukocytes were determined. Additionally, cell-free BAL fluid supernatants of the individual 1.5 ml BAL fluid aliquots were collected and subsequently subjected to ELISA for determination of cytokines.

After collection of BAL, individual lobes from the same previously lavaged lungs were removed, dissected, and homogenized in 2 ml HBSS without supplements (PAA Laboratories, Coëble, Germany) using a tissue homogenizer (IKA, Staufen, Germany). Subsequently, CFU were determined by plating 10-fold serial dilutions on sheep blood agar plates followed by incubation of the plates at 37°C/5% CO2 for 18 h.

#### BAL and preparation of lung parenchymal tissue

Mice were euthanized with an overdose of isoflurane (Baxter). Collection of BAL for the isolation of resident alveolar macrophages and alveolar recruited leukocytes from mice of the various treatment groups was done as described in detail recently (27, 28, 31, 32). Quantification of BAL fluid neutrophils, eosinophils, and lymphocytes was done on differential cell counts of Pappenheim-stained cytocentrifuge preparations, using overall morphological criteria, including cell size and shape of nuclei and subsequent multiplication of those values by the respective absolute BAL cell counts (27, 28, 31, 32). Quantification of resident and recruited mononuclear phagocyte subsets (alveolar macrophages, alveolar dendritic cells, and exudate macrophages) recovered by BAL from the lungs of mice from the various treatment groups was done using FACS-based differences in immunophenotypic profiles as characterized by differences in their cell surface Ag expression profiles of F4/80, CD11b, CD11c, and MHC-II, as outlined below in detail and elsewhere (27).

To quantify CD11c+ mononuclear phagocyte subsets in lung parenchymal tissue of mice of the various treatment groups, animals were subjected to BAL as described, followed by careful perfusion of the lungs via the right ventricle with HBSS until they were visually free of blood. Lung lobes were carefully removed and digested with collagenase A and DNase I. Leukocyte subsets contained in lung homogenates were further purified using a CD11c MACS kit following the instructions of the manufacturer (Miltenyi Biotec) and as previously described (28, 33, 34). The purity of isolated CD11c+ cells was always >90%. CD11c+ cells were then subjected to FACS analysis of differential cell surface Ag expression profiles, as outlined below.

#### Immunophenotypic analysis of mononuclear phagocyte subsets in BAL and lung parenchymal tissue

CD11c+ mononuclear phagocyte subsets isolated from lung parenchyma and BAL fluid were immunophenotypically analyzed according to their cell surface Ag expression profiles. Briefly, after preincubation with Octagam, 2–5 × 106 cells were stained with various combinations of
fluorochrome-conjugated mAbs directed against the corresponding cell surface markers for 20 min at 4°C. Subsequently, cells were washed twice with FACS buffer at 1200 rpm for 3 min at 4°C and cell acquisition was performed on a FACS Canto flow cytometer (BD Biosciences), equipped with an argon ion laser operating at 488 nm excitation wavelength and a helium neon laser operating at 633 nm excitation wavelength. First, the respective CD11c+ mononuclear phagocyte subsets purified from lung homogenates and BAL fluids of mice of the various treatment groups were gated according to their forward scatter (FSC) area versus side scatter (SSC) area characteristics and FSC area versus autofluorescence properties. Highly autofluorescent cells were further characterized as lung macrophages (F4/80+/CD11b+/CD11c+/MHC-II+) or as autofluorescent exudate macrophages characterized by their increased CD11b expression profile when compared with resident alveolar macrophages (F4/80+/CD11b+CD11c+/MHC-II-low). Data analysis and careful postacquisition compensation of spectral overlaps between the various fluorescence channels were performed using BD FACSDiva software.

Analysis of apoptosis/necrosis

Analysis of apoptosis and necrosis induction in alveolar macrophages and neutrophils was done by incubation of BAL cells with allophycocyanin-labeled annexin V (for determination of apoptosis) in the presence of propidium iodide (for the determination of necrosis) for 15 min at room temperature, according to the manufacturer’s instructions (BD Biosciences). Subsequently, macrophages were gated according to their FSC/SSC and FSC/F4/80 cell surface expression, and neutrophils were gated according to their FSC/SSC and GR-1 cell surface expression followed by determination of the percentage of apoptotic or secondary necrotic macrophages and neutrophils (28, 33).

Flow sorting of alveolar macrophages and neutrophils

A high-speed FACS Aria II flow cytometer (BD Biosciences) equipped with an argon ion laser operating at 488 nm excitation wavelength and an aerosol management system was used for sorting of alveolar macrophages and neutrophils from S. pneumoniae-infected mice therapeutically treated with GM-CSF. Briefly, BAL fluids were spun at 1400 rpm for 10 min at 4°C, and cell pellets were resuspended in FACS buffer. After preincubation with Octagam, 2–5 × 10^5 cells were stained with various combinations of fluorochrome-conjugated mAbs directed against the corresponding cell surface markers for 20 min at 4°C. Subsequently, cells were washed twice with FACS buffer at 1200 rpm for 3 min at 4°C and were resuspended for sorting in RPMI 1640. A BD FACS Aria II high-speed cell sorter was prepared for aseptic sorting according to the manufacturer’s instructions.

**FIGURE 1.** Effect of lung-specific overexpression of GM-CSF on pulmonary leukocyte recruitment. Mice were treated with either control vector or Ad-GM-CSF (1 × 10^8 PFU/mouse) for 1, 3, 5, 7, 10, 14, 21, or 28 d. At indicated time points, mice were subjected to BAL and lungs were removed for isolation of lung mononuclear phagocyte subsets. GM-CSF levels were determined in BAL fluids by ELISA (A). Recruitment profiles of resident alveolar macrophages (B) or newly recruited alveolar exudate macrophages in BAL fluids (C), or resident or recruited lung macrophages (D,E), as well as BAL fluid neutrophils (F) and lymphocytes (G), were determined. Values are shown as means ± SEM for n = 5–9 mice per time point and treatment group. *p < 0.05, **p < 0.01, ***p < 0.001 relative to control vector-treated mice.
Alveolar macrophages and neutrophils were gated according to their FSC area versus SSC area characteristics and FSC area versus autofluorescence properties. Alveolar macrophages were further gated according to their characteristic expression of the following cell surface markers: F4/80+/CD11c+/CD11b^2. Neutrophils were gated according to their GR-1+/CD11b^+ cell surface expression profile. After appropriate gating and compensation setting, cells were sorted at a flow rate of ~10,000 particles per second with an 85-μm nozzle and sample agitation turned on. The complete sorting process (presorting BAL fluid samples and postsorting macrophages or neutrophils) was performed at a constant temperature of 4°C and was finished with resort analysis of sorted cells to verify sort purities, which were always >98% (34).

**FIGURE 2.** Pulmonary PU.1 expression and STAT 5 phosphorylation in the lungs of mice transiently overexpressing GM-CSF. Mice were treated with either control vector or Ad-GM-CSF (1 × 10^8 PFU/mouse) for 3, 10, 14, or 28 d, as indicated. Subsequently, mice were sacrificed and immunoblot analysis of PU.1 expression or STAT5 phosphorylation was performed in whole lung lysates (A, B) or in lysates of alveolar macrophages 14 d after adenoviral transfer of GM-CSF (C). β-actin was used to control for similar protein loading of the gel (A, C). B, Fold change of STAT5 phosphorylation relative to unphosphorylated STAT5 in Ad-GM-CSF versus control vector-pretreated mice. The Western blots shown are representative of three independently performed experiments.

**FIGURE 3.** Survival and bacterial pathogen elimination in Ad-GM-CSF–treated mice challenged with *S. pneumoniae*. Mice were pretreated with either control vector or Ad-GM-CSF (1 × 10^8 PFU/mouse). At day 14 mice were infected with serotype 19 *S. pneumoniae* (~1.3 × 10^7 CFU/mouse; n = 10 mice/group) (A) or were infected with *S. pneumoniae* (~1.1 × 10^7 CFU/mouse; n = 10 mice/group) at day 28 (B). The time point of pneumococcal infection is indicated as day 0 (A, B) and corresponds to day 14 (A) or 28 (B) after control vector or Ad-GM-CSF treatment. Survival was monitored during an observation period of 10 d. In separate experiments, bacterial loads were determined in BAL fluids and lung parenchymal tissue of mice pretreated for 14 or 28 d with Ad-GM-CSF or control vector prior to infection with serotype 19 *S. pneumoniae* (~1 × 10^7 CFU/mouse), as indicated (C, D). Values are shown as means ± SEM for n = 5–9 mice per time point and treatment group. *p < 0.05, **p < 0.01 relative to control vector treated mice.
Total cellular RNA isolation, cDNA synthesis, and real-time RT-PCR

Total cellular RNA was isolated from flow-sorted, highly purified (>98%) alveolar macrophages and neutrophils from S. pneumoniae-infected and GM-CSF–treated mice 6 h postinfection or from control alveolar macrophages sorted from vehicle or GM-CSF–treated mice using a commercially available RNasey Micro Kit (Qiagen, Hilden, Germany), following the instructions of the manufacturer. Sorted alveolar macrophages or neutrophils (150,000) were lysed, and from the resultant RNA preparations, 100 ng total RNA was used for cDNA synthesis. Real-time RT-PCR analysis was performed as described recently (34, 35). For normalization, β-actin (forward primer, 5′-CCACAGTCAAGGGAATACG-3′; reverse primer, 5′-TCCTCAGGCGAGGAAATCATG-3′) was used as the housekeeping gene and mean fold changes were calculated using the 2−ΔΔCt method (34, 36). Primers used for determination of iNOS expression in sorted alveolar macrophages and neutrophils were: forward primer, 5′-GCCACCCAACATGGCAACA-3′; reverse primer, 5′-CGTACCGGATGACTGTGACTAATGTTT-3′.

Western blot analysis

For western blot analysis of PU.1, STAT5, phospho-STAT5, iNOS, cleaved caspase-3, caspase-3, and GAPDH protein expression in lung tissue, lungs were removed and homogenized on dry ice. The resulting lung tissue homogenate was passed through a cell strainer and transferred to ice-cold lysis buffer containing protease inhibitors. Similar to lung homogenates, CD11c+ lung mononuclear phagocytes and alveolar macrophages were lysed in ice-cold lysis buffer (100 μl/12 × 106 cells) as described previously (37). Expression analysis of phospho-STAT5, STAT5, PU.1, iNOS, cleaved caspase-3, caspase-3, and β-actin, and GAPDH was performed using specific Abs against these proteins as specified above. Expression of immunogenic proteins was determined by evaluation of ECL signals (ECL Plus; GE Healthcare, Buckinghamshire, U.K.) using a Vilber/Lourmat Chemi-Smart 5000 and STAT5 phosphorylation, and iNOS protein expression was further quantified using the Bio1D software package (Vilber/Lourmat Deutschland, Eberhardzell, Germany).

Phagocytosis and bacterial killing assay

Alveolar macrophages (2 × 105/well) were infected with serotype 19 S. pneumoniae at a multiplicity of infection of 50 for 30 min in RPMI 1640/10% FCS plus 1% glutamine at 37°C/5% CO2. Subsequently, non-phagocytized pneumococci were removed by four washing steps with HBSS, and either vehicle (PBS) or 100 ng/ml rmGM-CSF dissolved in RPMI 1640 was added to each well followed by incubation at 37°C/5% CO2. Sixty and 120 min later, alveolar macrophages were washed and lysed with 0.1% saponin in HBSS to release intracellular pneumococci. Subsequently, CFU were quantified by plating of 10-fold serial dilutions of cell lysates on sheep blood agar plates followed by incubation of the plates at 37°C/5% CO2 for 18 h. In control experiments, S. pneumoniae-infected alveolar macrophages were incubated in the presence of gentamicin (20 μg/ml) to exclude CFU determinations to be affected by extracellular pneumococci.

Lung histopathology

Mice were euthanized, and nonlavaged whole lungs were harvested and immediately submitted to immersion fixation in buffered formalin. After routine embedding, two whole lung sections from each lung lobe were cut (3-μm sections), stained with H&E, and analyzed by an experienced lung pathologist (F.L.) (5) blinded to the respective pretreatment regimens. For all specimens, the composition and distribution of the inflammatory infiltrate, if present, were analyzed. The presence of lymphocytes, granulocytes, and macrophages was semiquantitatively scored in 10% increments and their allocation in alveolar spaces, alveolar walls, and peribronchial or perivascular tissue was noted. The percentage of bronchovascular bundles with significant inflammatory infiltrates was recorded as well as the density of the infiltrate using a four-tier score (0, no infiltrate; 1, lymphocytic cuffs less than five cell layers thick; 2, thicker lymphocytic cuffs than five cell layers; and 3, spillover of lymphocytes into the alveolar walls). Furthermore, the presence of bronchopneumonia (florid intra-alveolar granulocytic infiltrate) or a pattern of organizing pneumonia was noted.

FIGURE 4. Alveolar leukocyte recruitment profiles in Ad-GM-CSF–pretreated mice subsequently infected with S. pneumoniae. Mice were pretreated with either control vector or Ad-GM-CSF (1 × 108 PFU/mouse) for 14 (A–C) or 28 d (D–F) prior to infection with serotype 19 S. pneumoniae (1 × 107 CFU/mouse). At indicated time points, mice of the respective treatment groups were subjected to BAL for determination of resident alveolar macrophages (A, D), newly recruited exudate macrophages (B, E), or neutrophils (C, F). G and H. Histopathological assessment of interstitial lung inflammation in H&E-stained lung tissue sections of mice either pretreated with control vector or Ad-GM-CSF vector for 7 (G) or 14 d (H) followed by infection with serotype 19 S. pneumoniae (1 × 107 CFU/mouse) for 7 d. Values are shown as means ± SEM for n = 5–9 mice per time point and treatment group. *p < 0.05, **p < 0.01 relative to control vector treated mice.
ELISA
Cytokine concentrations (GM-CSF, IL-6, TNF-α, KC, MIP-2) were determined in BAL fluids of S. pneumoniae-infected or control mice by using commercially available DuoSet ELISAs according to the manufacturer’s instructions (R&D Systems, Wiesbaden, Germany).

Statistical analysis
All data are given as means ± SEM. Differences between controls and respective treatment groups were analyzed by ANOVA followed by a post hoc Dunnett test. Significant differences between groups were analyzed by Levene’s test for equality of variances followed by a Student t test using an SPSS for Windows software package. CFU determinations were analyzed using a Mann–Whitney U test. Survival curves were compared by a log-rank test. Statistically significant differences between treatment groups were assumed when p values were <0.05.

Results
Overexpression of GM-CSF triggers transient leukocyte recruitment into the lungs of mice
First, we characterized GM-CSF protein levels in BAL fluid in response to adenoviral gene transfer of murine GM-CSF or empty control vector. As shown in Fig. 1A, adenoviral gene transfer of GM-CSF rapidly increased GM-CSF protein levels in BAL fluids with peak by day 3, and significantly increased GM-CSF protein levels were still detectable in BAL fluids until day 28. No GM-CSF was detectable in BAL fluids of control vector-treated animals (Fig. 1A).

Next, we analyzed the impact of transient GM-CSF overexpression on pulmonary leukocyte recruitment by determining changes in the leukocyte population in BAL fluid. As shown in Fig. 1B–E, recruitment of exudate macrophages into the broncho-alveolar and lung parenchymal compartment was substantially increased in mice receiving Ad-GM-CSF compared with control vector-treated mice, reaching significantly increased numbers by day 5 until day 28 posttreatment (Fig. 1C, 1E). Moreover, intra-alveolar GM-CSF release also led to increased numbers of neutrophils (Fig. 1F) and lymphocytes (Fig. 1G) accumulating in the alveoli, with peak cell numbers noted by days 7–10 (Fig. 1F, 1G), whereas numbers of eosinophils in BAL fluid of Ad-GM-CSF–treated mice were only marginally elevated (data not shown). These changes in the alveolar leukocyte recruitment profile were accompanied by increased expression of IL-6 and KC in BAL fluids of mice receiving Ad-GM-CSF relative to control vector-treated mice (Supplemental Fig. 1A, 1B), whereas Ad-GM-CSF did not lead to increased expression of TNF-α, CCL2, IFN-γ, and IL-12 in BAL fluids of mice (data not shown). No GM-CSF was detectable in the serum of Ad-GM-CSF–treated mice, and no significant differences were observed in blood neutrophil and lymphocyte counts or numbers of resident or inflammatory monocyte subsets between Ad-GM-CSF–treated and control vector-treated mice (data not shown).

Transient intra-alveolar overexpression of GM-CSF triggers sustained STAT5 phosphorylation and increased PU.1 protein levels in lung tissue and alveolar macrophages
Western blot analysis of whole lung tissue demonstrated increased PU.1 protein expression and increased STAT5 phosphorylation starting as early as day 3 up to day 28 following GM-CSF gene transfer (Fig. 2A, 2B). To determine the role of alveolar macrophages in this process, alveolar macrophages were flow-sorted from BAL fluid of Ad-GM-CSF– or control vector-treated mice and their STAT5 phosphorylation and PU.1 protein levels were determined. As shown in Fig. 2C, GM-CSF overexpression resulted in increased PU.1 expression and STAT5 phosphorylation in alveolar macrophages by day 14. FACS analysis of alveolar macrophages from Ad-GM-CSF–treated mice demonstrated up-regulation of TLR2 and CD11b cell surface expression, both of which are known to be dependent on functional PU.1 (data not shown). Collectively, these data show that intratracheal Ad-GM-CSF delivery leads to sustained increased PU.1 expression and STAT5 signaling in lung tissue of mice (up to 28 d), which is associated with increased GM-CSF–dependent accumulation and activation of alveolar and lung macrophages (Fig. 2A, 2B).

Effect of transient pulmonary overexpression of GM-CSF on lung protective immunity against focal pneumonia-inducing S. pneumoniae strain
To determine whether prophylactic administration of Ad-GM-CSF is able to enhance protective immunity against S. pneumoniae infections, mice were treated with Ad-GM-CSF or control vector followed by infection with focal pneumonia-inducing serotype 19 S. pneumoniae on days 3, 14, or 28 after gene delivery. Mice that were infected with S. pneumoniae on day 3 after Ad-GM-CSF exhibited significantly reduced bacterial loads in BAL fluids and lungs at day 3 postinfection (CFU in BAL fluids of control vector-
treated mice: $7.3 \times 10^5 \pm 3.7 \times 10^5$ CFU versus Ad-GM-CSF-treated mice: $1.4 \times 10^7 \pm 9.5 \times 10^6$ CFU; $n = 6; p = 0.03$; CFU in lung tissue of control vector-treated mice: $1.2 \times 10^7 \pm 6.0 \times 10^6$ CFU versus Ad-GM-CSF-treated mice: $2.0 \times 10^7 \pm 1.7 \times 10^5$ CFU; $n = 6; p = 0.02$). Importantly, mice infected with *S. pneumoniae* at day 14 after Ad-GM-CSF treatment had an overall survival of 90%, whereas the survival of control vector-treated mice was just 10% (Fig. 3A, $p < 0.01$). Importantly, infection of mice with serotype 19 *S. pneumoniae* at day 28 after GM-CSF delivery resulted in 100% survival relative to ~50% survival noted in control vector-treated mice (Fig. 3B, $p < 0.05$).

Increased survival in serotype 19 *S. pneumoniae*-infected mice was accompanied by significantly improved pneumococcal clearance on days 1 and 3 postinfection in BAL fluids and lungs of mice pretreated with Ad-GM-CSF for 14 or 28 d (Fig. 3C, 3D). Closer examination of leukocytic responses revealed that mice infected with *S. pneumoniae* at day 14 after Ad-GM-CSF had significantly increased numbers of alveolar macrophages and exudate macrophages on days 1 and 3 postinfection, respectively (Fig. 4A, 4B), whereas neutrophil recruitment profiles at the same time were strongly reduced on days 1 and 3 postinfection (Fig. 4C). Mice infected with *S. pneumoniae* on day 28 after Ad-GM-CSF showed overall similar numbers of alveolar macrophages on days 1 and 3 postinfection (Fig. 4D). However, numbers of exudate macrophages were significantly increased on day 3 (Fig. 4E), whereas recruited neutrophils were significantly reduced in the alveolar lumen by days 1 and 3 postinfection (Fig. 4F). Expression of proinflammatory CXC cytokines KC and MIP-2 as well as IL-6 and TNF-α were significantly reduced in *S. pneumoniae*-infected mice pretreated with Ad-GM-CSF at day 14 or 28 prior to infection (Supplemental Fig. 2). Notably, further histopathological examination of lung tissue revealed substantially reduced interstitial inflammation in mice pretreated with Ad-GM-CSF for 7 or 14 d followed by infection with *S. pneumoniae* for 7 d (Fig. 4G, 4H). Collectively, these data demonstrate that pulmonary overexpression of GM-CSF improves lung innate immunity against noninvasive *S. pneumoniae*, suggesting a major role for intra-alveolar GM-CSF in enhancing local rather than systemic host defense mechanisms.

**FIGURE 6.** Prophylactic and therapeutic treatment of *S. pneumoniae*-infected mice with rGM-CSF. For prophylactic GM-CSF treatment, mice were treated orotracheally with either vehicle (open bars) or rGM-CSF (10 μg/mouse, filled bars) for 3, 6, or 12 h prior to infection with serotype 19 *S. pneumoniae* ($5 \times 10^5$ CFU/mouse) (A, B). For therapeutic GM-CSF treatment, mice were infected with serotype 19 *S. pneumoniae* ($5 \times 10^5$ CFU/mouse) followed by therapeutic application of either vehicle or rGM-CSF (20 μg/mouse) applied at 1.5, 3, 6, or 12 h postinfection (C, D). At 24 h postinfection, mice were subjected to BAL and bacterial loads (A, C), or newly recruited exudate macrophages (B, D) were quantified in BAL fluids. Values are shown as means ± SEM for *n* = 5–9 mice per time point and treatment group. *p < 0.05, **p < 0.01, ***p < 0.001 relative to vehicle treated mice.

Lung-specific overexpression of GM-CSF protects pulmonary macrophages and neutrophils from *S. pneumoniae*-induced apoptosis

Infections of the lung with *S. pneumoniae* are known to rapidly induce apoptosis in resident alveolar macrophages within 24 h, thereby transiently depleting alveolar macrophages from distal airspaces (5, 27, 28, 30, 38–40). Therefore, we examined whether pretreatment of mice with Ad-GM-CSF would protect lung macrophages from *S. pneumoniae*-induced apoptosis. As shown in Fig. 5, transfer of the GM-CSF gene 14 or 28 d prior to infection with *S. pneumoniae* significantly reduced the percentage of primary apoptotic macrophages (annexin V+) as well as secondary necrotic macrophages (annexin V+ and propidium iodide+) in BAL fluids 24 h postinfection (Fig. 5A–D). No significant differences were observed in the percentage of necrotic macrophages between both groups (data not shown). Additionally, the percentage of apoptotic neutrophils, but not secondary necrotic neutrophils, was significantly reduced in BAL fluids of mice pretreated with Ad-GM-CSF for 28 d followed by 24 h pneumococcal challenge (Fig. 5E, 5F). No differences were observed in the percentage of apoptotic and secondary necrotic neutrophils in *S. pneumoniae*-challenged mice pretreated with Ad-GM-CSF for 14 d (data not shown).

**Prophylactic and therapeutic delivery of mice with rGM-CSF improves lung innate immunity against *S. pneumoniae***

After having demonstrated a protective role for Ad-GM-CSF against pulmonary infections with noninvasive *S. pneumoniae*, we examined whether intratracheal application of rGM-CSF would also improve antibacterial responses in the lungs of mice. We first treated mice orotracheally with GM-CSF (10 μg/mouse) at 3, 6, and 12 h prior to infection with noninvasive serotype 19 *S. pneumoniae*. As shown in Fig. 6, we found significantly decreased bacterial loads in BAL fluids of GM-CSF–pretreated mice compared with vehicle-treated mice at 24 h postinfection with *S. pneumoniae* (Fig. 6A). At the same time, numbers of exudate macrophages were increased in GM-CSF–pretreated mice compared with vehicle-treated mice 24 h postinfection (Fig. 6B), whereas no significant differences were observed in numbers of alveolar neutrophils as was observed (Supplemental Fig. 3A). Notably, s.c. pretreatment of mice...
for 6 d with GM-CSF (10 μg/mouse/d) followed by pneumococcal challenge did not show any differences in bacterial clearance and pulmonary leukocyte recruitment relative to vehicle-treated mice (data not shown), suggesting that intratracheal rather than systemic delivery of GM-CSF is important to enhance lung-protective immunity against inhaled bacterial pathogens.

Next, we examined the therapeutic potential of intratracheally applied GM-CSF to improve lung innate immunity against S. pneumoniae infections in mice. Therapeutic treatment of mice with rmGM-CSF (20 μg/mouse) applied at 1.5, 3, 6, or 12 h after pneumococcal infection led to significantly increased pneumococcal clearance at 24 h (Fig. 6C) and 48 h postinfection (data not shown). Therapeutic treatment of pneumococcal pneumonia with GM-CSF was most effective when initiated at 3–6 h postinfection with S. pneumoniae (Fig. 6C), resulting in significantly reduced bacterial loads in BAL fluids of mice analyzed at 24 h post pneumococcal infection (Fig. 6C) and even at 48 h after pneumococcal infection (vehicle application at 3 h postinfection: 2.7 × 10^6 ± 2.6 × 10^5 CFU in BALF; GM-CSF application at 3 h postinfection: 9.3 × 10^6 ± 5.7 × 10^5 CFU in BALF; p < 0.05).

Under these experimental conditions, we observed significantly increased GM-CSF protein levels in BAL fluids of mice after 24 h and even 48 h after pneumococcal infection (24 h postinfection: vehicle-treated mice: 0.01 ± 0.006 ng/ml; GM-CSF–treated mice: 59.8 ± 17.0 ng/ml; 48 h postinfection: vehicle-treated mice: 0 ng/ml; GM-CSF–treated mice: 1.2 ± 0.5 ng/ml). At the same time, numbers of exudate macrophages were significantly increased in mice treated with GM-CSF at all investigated time points after pneumococcal infection (Fig. 6D), whereas GM-CSF therapy of pneumococcal pneumonia did not induce increased neutrophilic alveolitis in mice (Supplemental Fig. 3B).

**Treatment of mice with GM-CSF triggers increased iNOS expression and antibacterial activity in alveolar macrophages**

To elucidate whether GM-CSF–induced activation of alveolar macrophages directly improved their bactericidal functions, we analyzed iNOS gene expression in flow-sorted macrophages and neutrophils collected from the lungs of S. pneumoniae-infected mice therapeutically treated with GM-CSF. As shown in Fig. 7, iNOS mRNA levels (Fig. 7B) and protein expression (Fig. 7C) were significantly increased in sorted macrophages of S. pneumoniae-infected mice treated with GM-CSF at 3 h postinfection. Notably, treatment of mice with GM-CSF increased iNOS mRNA levels in sorted macrophages by 6-fold relative to alveolar macrophages of vehicle-treated, S. pneumoniae-infected mice (Fig. 7B). Moreover, sorted macrophages showed a strongly increased upregulation of iNOS mRNA relative to sorted neutrophils from GM-CSF–treated mice (Fig. 7A). In vitro pneumococcal killing assays with primary alveolar macrophages revealed a significantly improved bacterial killing at 60 and 120 min postinfection in the presence of rGM-CSF (100 ng/ml) (Fig. 7D). Collectively, these data show that therapeutic treatment of S. pneumoniae-infected mice with GM-CSF particularly triggers increased iNOS expression in alveolar macrophages but not neutrophils, and it induces increased bactericidal activities in alveolar macrophages, thereby contributing to GM-CSF–induced improved lung innate immunity to this prototypic lung bacterial pathogen.

**Treatment of mice with GM-CSF protects alveolar macrophages from S. pneumoniae-induced apoptosis**

GM-CSF has been shown to be an important survival factor for leukocytes. Therefore, we next questioned whether therapeutic
GM-CSF was initially described in 1977 as a factor contained in the lungs of mice challenged with S. pneumoniae. With increased GM-CSF bioavailability in the lungs, the leukocytic recruitment profiles shifted from a neutrophil-dominated toward a macrophage-rich phenotype during infection. This was accompanied by a substantially decreased release of proinflammatory cytokines and neutrophil chemoattractants, along with reduced apoptosis and secondary necrosis of macrophages. Reduced apoptosis rates were also observed in mice treated with rGM-CSF. Taken together, we show in this study that prophylactic administration of GM-CSF in a mouse model of pneumococcal pneumonia results in a shift from a neutrophil-dominated toward a macrophage-rich inflammatory response with long-lasting effects on bacterial clearance and survival. These data support a strong immunostimulatory effect of intra-alveolar GM-CSF augmenting lung innate immunity against bacterial pneumonia in mice.

GM-CSF as a pleiotropic growth factor and cytokine with a wide range of biological activities under normal and disease conditions (9). Studies in GM-CSF knockout and transgenic mice revealed the critical importance of GM-CSF in surfactant homeostasis and its importance in antibacterial defense against a wide range of bacterial, viral, and fungal pathogens (14–24, 45). Similar to G-CSF, GM-CSF is frequently used as adjuvant therapy in patients with hematological malignancies to augment bone marrow stem cell engraftment and expansion subsequent to whole body irradiation (46). GM-CSF is further employed as a valuable adjuvant therapy to compensate for autoantibody-induced endogenous GM-CSF neutralization in patients with pulmonary alveolar proteinosis (47–49). More recently, delivery of recombinant human GM-CSF by inhalation was found to be effective for the treatment of human pulmonary alveolar proteinosis, and at the same time was also well tolerated by healthy control individuals (50). Only very limited data are available that characterize the usefulness of prophylactic GM-CSF as a novel approach to augment antibacterial host defense mechanisms, particularly in immunocompromised patients (45). Individuals with increased risk of acquiring lung bacterial infections include HIV patients, patients with hematological malignancies undergoing a whole body irradiation regimen, or lung transplant recipients. To date, preventive strategies are limited to vaccination approaches against major lung pathogens such as S. pneumoniae, with clinical protection rates of no more than 60–70%. This suboptimal situation underscores the need for novel adjuvant therapies. Therefore, we evaluated whether prophylactic GM-CSF has the potential to enhance protective immunity against pneumococcal pneumonia in a mouse model. We found that 90–100% of the mice survived otherwise lethal pneumococcal pneumonia when pretreated with Ad-GM-CSF 14 and 28 d prior to infection with S. pneumoniae. Prophylactic administration of rGM-CSF was only effective in improving lung innate immunity against pneumococcal pneumonia when given by inhalation, whereas systemic delivery had no protective effects in our studies. The protective efficacy of inhaled GM-CSF against pneumococci was limited to those serotypes causing localized focal pneumonia.
(serotype 19) but did not prevent the development of invasive pneumococcal disease caused by sepsis-inducing serotype 2 S. pneumoniae (data not shown). This finding may be due to different time kinetics by which intratracheally delivered GM-CSF triggers antibacterial activities in professional phagocytes relative to the short time that serotype 2 S. pneumoniae needs to cause invasive pneumococcal disease. Consistent with our experimental data, a clinical trial in preterm neonates also showed that systemic administration of GM-CSF is not able to reduce sepsis rates or improve survival (51). In contrast, i.v. infusion of GM-CSF in patients with severe sepsis and respiratory failure significantly improved oxygenation and had a positive impact on the development of adult respiratory distress syndrome, which was associated with improved function of blood and alveolar neutrophils (52). This suggests that GM-CSF is most effective when administered directly to the site of infection, which may explain why systemic delivery of GM-CSF was not protective in mice with pneumococcal pneumonia. Further studies are needed to evaluate the efficacy of i.v. GM-CSF on sepsis rates in mice infected with sepsis-inducing, highly virulent serotype 2 S. pneumoniae.

We observed that pulmonary overexpression of GM-CSF shifted the leukocyte recruitment profiles from a neutrophil-dominated toward a macrophage-dominated phenotype during infection with S. pneumoniae. This shift was characterized by reduced release of proinflammatory cytokines and chemokines along with decreased numbers of apoptotic and secondary necrotic macrophages. Additionally, prophylactic overexpression of GM-CSF prior to pneumococcal infection substantially reduced interstitial lung inflammation and neutrophilic alveolitis subsequent to pneumococcal challenge. Both secondary necrotic macrophages and secondary necrotic neutrophils contribute to increased systemic inflammatory responses to bacterial challenge, which in turn promotes lung tissue damage and increased death in mice and humans (5, 27, 53). Thus, we think that the substantial GM-CSF-mediated inhibition of apoptosis and secondary necrosis in alveolar macrophages contributes to the overall dampened proinflammatory immune responses resulting in overall increased survival of pneumococcal pneumonia in our model.

In the present study, we observed a macrophage-dominated lung innate immune response to pneumococcal pneumonia in mice prophylactically exposed to Ad-GM-CSF, resulting in increased survival. In this regard, we and others (5, 54) recently reported that in a condition where exudate macrophages accumulate in the alveolar space, for example, in CCL2 transgenic mice, lung bacterial loads are substantially reduced, along with dampened pulmonary inflammation and improved survival (5). Additionally, Schabauer and colleagues recently demonstrated in myeloid cell PTEN-deficient mice that reduced numbers of lung-infiltrating neutrophils were offset by enhanced phagocytic properties of alveolar macrophages, resulting in improved bacterial clearance and augmented resolution of inflammation in response to S. pneumoniae (54). Whether or not the shift from a neutrophil- toward a macrophage-dominated immune response as observed in GM-CSF-pretreated or PTEN knockout mice represents a principal mechanism by which lung pathology caused by excessive inflammatory response following bacterial infections might be attenuated to improve survival warrants further investigation.

In the present study, adenalvalve vectors were just employed as an efficient gene delivery system with specificity for bronchial and alveolar epithelial cells, leading to strong transgene expression specifically within the bronchoalveolar compartment of mice. Clinically, prophylactic inhalation of GM-CSF might be an effective adjuvant in the management of pulmonary infections with multidrug-resistant bacterial pathogens, where GM-CSF therapy could be used in combination with newer antibiotics such as daptomycin, which per se has limited antibacterial efficacy in the bronchoalveolar space of mice and humans (55). Repetitive inhaled GM-CSF applications could be considered for stimulation of innate immune responses in patients at risk for acquiring bacterial infections (50). We found prophylactic intra-alveolar deposition of GM-CSF to exert a long-lasting protection against pneumococcal pneumonia, whereas therapeutic administration of rGM-CSF was less effective when initiated at later time points postinfection. Therefore, we think that particularly prophylactic application of GM-CSF might be useful for prevention of pneumococcal pneumonia in immunocompromised patients.

In summary, to our knowledge, we show for the first time that prophylactic delivery of GM-CSF triggers long-lasting immunostimulatory effects in the murine lung and protects mice from lethal pneumococcal pneumonia. These data may direct the development of novel antibiotic-independent immunostimulatory therapies to protect immunocompromised patients against bacterial pneumonias.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


GM-CSF IN PNEUMOCOCCAL PNEUMONIA


