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Cathepsin G and Neutrophil Elastase Contribute to Lung-Protective Immunity against Mycobacterial Infections in Mice

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The neutrophil serine proteases cathepsin G (CG) and neutrophil elastase (NE) are involved in immune-regulatory processes and exert antibacterial activity against various pathogens. To date, their role and their therapeutic potential in pulmonary host defense against mycobacterial infections are poorly defined. In this work, we studied the roles of CG and NE in the pulmonary resistance against Mycobacterium bovis bacillus Calmette-Güerin (BCG). CG-deficient mice and even more pronounced CG/NE-deficient mice showed significantly impaired pathogen elimination to infection with M. bovis BCG in comparison to wild-type mice. Moreover, granuloma formation was more pronounced in M. bovis BCG-infected CG/NE-deficient mice in comparison to CG-deficient and wild-type mice. A close examination of professional phagocyte subsets revealed that exclusively neutrophils shuttled CG and NE into the bronchoalveolar space of M. bovis BCG-infected mice. Accordingly, chimeric wild-type mice with a CG/NE-deficient hematopoietic system displayed significantly increased lung bacterial loads in response to M. bovis BCG infection. Therapeutically applied human CG/NE encapsulated in liposomes colocalized with mycobacteria in alveolar macrophages, as assessed by laser scanning and electron microscopy. Importantly, therapy with CG/NE-loaded liposomes significantly reduced mycobacterial loads in the lungs of mice. Together, neutrophil-derived CG and NE critically contribute to deceleration of pathogen replication during the early phase of antimycobacterial responses. In addition, to our knowledge, we show for the first time that liposomal encapsulated CG/NE exhibit therapeutic potential against pulmonary mycobacterial infections. These findings may be relevant for novel adjuvant approaches in the treatment of tuberculosis in humans. The Journal of Immunology, 2012, 188: 000-000.

Tuberculosis remains a global health threat. Approximately 8.8 million people are newly infected with Mycobacterium tuberculosis, and ∼1.4 million people die annually from tuberculosis (1). In the mouse model, the early phase of mycobacterial infections of the lung is characterized by nearly exponential growth of mycobacteria within alveolar macrophages (2). Control of mycobacterial replication is initiated by adequate T cell-dependent activation of macrophages to overcome mycobacteria-induced arrest of phagosomal maturation (3, 4).

Professional phagocytes are a heterogeneous group of phagocytic cells of myeloid origin, including neutrophils and mononuclear phagocytes, which subdivide in organ-specific macrophages and myeloid dendritic cell (DC) subsets. These cells are differentially equipped with an array of lysosomal proteolytic and antimicrobial effector molecules, including cysteine, aspartate, and serine proteases (cathepsins), as well as non-cathepsin serine proteases like neutrophil elastase (NE) and proteinase-3, and various defensins (3, 5). The two neutrophil serine proteases cathepsin G (CG) and NE are classically expressed in azurophilic granules of neutrophils, where they are stored in their active form. Both serine proteases are synthesized as zymogens and are activated after cleavage and removal of the N-terminal prosesequence of two amino acid residues (6). Besides their involvement in various biological processes like degradation of extracellular matrix components or the regulation of immune responses through processing of chemokines and cytokines, CG and NE exert antimicrobial activity against various pathogens (6, 7). It has been shown that CG is implicated in the antimicrobial defense against Staphylococcus aureus, and, together with NE, also against Aspergillus fumigatus infection in mice (8, 9). Recently, work of our group showed that neutrophil-derived CG and NE are crucial for effective lung innate immune responses against Streptococcus pneumoniae infections in...
mice (10). In line with this finding, CG, NE, and proteinase-3 were recognized to contribute to human neutrophil killing of S. pneumoniae in vitro (11). In addition, NE is important for the elimination of Klebsiella pneumoniae and Escherichia coli as well as Candida albicans and Pseudomonas aeruginosa (8, 12–14).

In contrast, the role of CG and neutral serine protease NE in chronic infections of the lung elicited by mycobacterial pathogens is poorly defined. We recently demonstrated that among various cathespins, particularly CG, but not cathepsin K, L, S, D, or B, mRNA was strongly upregulated in Mycobacterium bovis bacillus Calmette-Guérin (BCG)-infected alveolar macrophages in mice at 7 d post infection. CG expression triggered in vivo by lung mycobacterial infections may therefore be part of an endogenous host defense program against mycobacteria (15). This suggestion is further supported by a recent study demonstrating that in NO synthase 2-deficient mice CG contributes to control of M. tuberculosis in hypoxic lung granulomas in mice (16). In contrast, previous work demonstrated that CG was downregulated in a human monocytic THP-1 cell line in response to M. tuberculosis in vitro, suggesting a possible escape mechanism of mycobacteria to overcome mycobacterial killing by macrophages (17). However, there are currently no data available to directly specify roles for CG and NE in the lung host defense against mycobacterial infections in vivo. Therefore, CG-deficient mice, CG/NE-deficient mice, and respective wild-type controls were infected via the airways with M. bovis BCG, and the course and severity of infection were analyzed. Our study provides evidence that CG and NE are involved in early containment of mycobacterial growth. Capitalizing on this finding, we then explored a novel therapeutic anti-mycobacterial approach by treating M. bovis BCG-infected mice with CG and NE encapsulated in liposomes to achieve a targeted uptake of exogenously administered proteolytic activities specifically by alveolar macrophages, the primary target cells of mycobacterial infections. To our knowledge, the data show for the first time that therapeutically applied liposomal encapsulated human CG/NE colocalize with mycobacteria within the same compartment of alveolar macrophages and improve lung-protective immunity against mycobacterial infections.

Materials and Methods

Animals

Female control mice (129 S2/SvPasCrl) were initially purchased from Charles River Laboratories. CG-deficient mice and CG/NE double-knockout (KO) mice on a 129 S2 background were generated, as described elsewhere (9), and were obtained from the Medical Research Council (Oxford, U.K.). All animals were used for the described experiments at 8–12 wk of age, and matched for sex and genetic background. This study was carried out in strict accordance with the guidelines of the Animal Care and Use Committee of the Central Animal Facility at the Hannover School of Medicine. Animal experiments were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (Hannover, Germany).

Reagents

Purified CG from human sputum was purchased from Sigma-Aldrich (Steinheim, Germany) and Biocentrum (Krakow, Poland). Purified cathepsin B (CtsB) from human liver was obtained from Calbiochem (Darmstadt, Germany). Cholesterol and egg phosphatidylcholine were purchased from Sigma-Aldrich (Steinheim, Germany) and Avanti Polar Lipids (Alabaster, AL), respectively. Goat anti-mouse CG polyclonal Ab (clone I-19) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-mouse β-actin mAb was obtained from Sigma-Aldrich (München, Germany; clone AC 15), and peroxidas-conjugated donkey anti-mouse polyclonal IgG (H+L) and peroxidase-conjugated goat anti-mouse polyclonal IgG (H+L) were 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 II PE, anti-Ly-6G/Ly-6C (GR-1) FITC, CD3 FITC, CD4 PerCP Cy5.5, CD8a allophycocyanin, CD10 PE, and CD45 PE Cy7, were purchased from BD Biosciences (Heidelberg, Germany). MACS kit and CD11c, CD4, CD8a, CD19, and Ly-6G beads were all purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). FITC-labeled BSA (FITC-BSA) and lipophilic PKH26-GL fluorescent cell linker kit were purchased from Sigma-Aldrich (Steinheim, Germany).

Preparation of liposome-encapsulated CG and NE

Liposome encapsulation of CG and NE was done as previously reported, with some modifications (18–20). Briefly, 4 mg cholesterol was added to 43 mg egg phosphatidylcholine, and the chloroform phase was evaporated with a rotary evaporator (Büchi, Flawil, Switzerland). The CG- and NE-containing solution was prepared by dissolving 3 mg of each enzyme in 2.5 ml sterile PBS, and this solution was added to the lipid film and mixed thoroughly. Empty liposomes were made by addition of PBS alone. This solution was sonicated and centrifuged at 13,000 rpm for 1 h at 4°C. The supernatant was discarded, and the liposomal pellet was resuspended twice in sterile PBS and centrifuged again. Subsequently, liposomes were resuspended in 2.5 ml sterile PBS, kept at 4°C, and used within 24 h after preparation. In selected experiments, liposomes were labeled with the lipophilic dye PKH26 (10 μM), according to the manufacturer’s instructions, or were loaded with FITC-BSA (1 or 5 mg) to control uptake of liposomes by alveolar macrophages in vivo using confocal laser-scanning microscopy and flow cytometric analysis, respectively.

Culture and quantification of M. bovis BCG

GFP-expressing M. bovis BCG (GFP-BCG) and M. bovis BCG (both strain Pasteur) were cultured in Middlebrook 7H9 medium until midlog phase (15) and then frozen at −80°C until use. For quantification, mycobacteria were serially diluted in Middlebrook 7H9 medium and plated on Middlebrook 7H10 agar plates. After 3 wk of incubation at 37°C, CFU were determined.

Infection of mice

Mice were anesthetized by i.m. application of tetracaine hydrochloride (2.5 mg/kg; Rompun, Bayer, Leverkusen, Germany) and ketamine (50 mg/kg; Ketamin Graüb, Albrecht, Aulendorf, Germany) followed by low-dose infection of mice with M. bovis BCG (2 × 103 CFU/mouse), which was done via intratracheal application, as described recently in detail (15).

Intratracheal instillation of LPS

Intratracheal instillation of LPS (20 μg/mouse) into the lungs of mice was performed in the same manner as described for the intratracheal instillation of M. bovis BCG. In selected experiments, mice received intratracheal applications of 20 μg LPS/mouse for 12−24 h to trigger neutrophil recruitment into the bronchoalveolar space. Subsequently, mice were euthanized, and elicited neutrophils (purity >95%) contained in bronchoalveolar lavage (BAL) fluids were collected for further experiments in vitro.

Intratracheal instillation of liposome-encapsulated CG and NE

At 24 h post-M. bovis BCG infection, wild-type mice were anesthetized with desflurane and received a single (day 1 postinfection) or a repetitive application (days 1 and 7 postinfection) of liposomal CG/NE (100 μl per mouse) or PBS-containing liposomes as control. Intratracheal instillation of liposome-encapsulated CG/NE into the lungs of mice was performed in the same manner as described for the intratracheal instillation of M. bovis BCG. In selected experiments, mice received intratracheal applications of 20 μg LPS/mouse for 12−24 h to trigger neutrophil recruitment into the bronchoalveolar space. Subsequently, mice were euthanized, and elicited neutrophils (purity >95%) contained in bronchoalveolar lavage (BAL) fluids were collected for further experiments in vitro.

BAL and preparation of lung, spleen, and lung draining lymph nodes for quantification of bacterial loads

BAL was performed, as described recently (15, 21, 22). Total BAL fluid cells were counted, followed by preparation of cytospins and Pappenheim staining used for determination of leukocyte subset differentials. Remaining BAL cells were lysed in HBSS containing 0.1% saponin (pH 7.2) for 10 min at 37°C, and then plated in 10-fold serial dilutions on Middlebrook 7H10 agar plates and incubated at 37°C for 3 wk for CFU determination. Lung lobes, spleen, and lung draining lymph nodes (dLN) were carefully isolated, while avoiding any contamination of organs with conducting airways. Lung and spleen tissue was homogenized using a tissue homogenizer (IKA, Staufen, Germany), the resulting homogenates were further diluted of lung or spleen homogenates were plated on 7H10 agar plates and incubated at 37°C for 3 wk for CFU determination. Determination of mycobacterial loads in lung dLN was done by lysis of the lung dLN in 0.1% saponin (pH 7.2) for...
10 min at 37°C, followed by incubation for 10 min in an ultrasonic bath. Afterward, lung dLN were crushed with a pistil, and homogenates were 10-fold serially diluted and plated onto 7H10 agar plates.

**Determination of antimycobacterial activity of CG**

For determination of the antimycobacterial activity of CG in vitro, M. bovis BCG cultures were grown to midlog phase, and 100-μl aliquots containing ~1 × 10^8 mycobacteria were incubated with human CG or human C3bB serving as a control at different concentrations (1, 10, 25, 50, or 100 μg/ml) for 72 h at 37°C both under aerobic or anaerobic conditions in 96-well plates. Maintenance of anaerobic culture conditions was achieved by using the anaerocult system (Merck, Darmstadt, Germany). Briefly, plates and anaerogen sachets (Oxoid, Cambridge, U.K.) were placed in an anaerobic jar (Merck). Anaero-test strips were fixed in the container to control for anaerobic conditions. Subsequently, the anaerobic jar was sealed and incubated at 37°C. After 72 h of culture, 10-fold serial dilutions of samples were prepared in 7H9 medium and plated onto 7H10 agar plates for determination of CFU.

**Isolation of peripheral blood monocytes, lung CD11c+ mononuclear phagocyte subsets, and lung neutrophils**

Anticoagulated blood collected from the vena cava of untreated or M. bovis BCG-infected mice was diluted 1:4 in PBS, layered on LymphoLyte (BIOZOL, Eching, Germany), and then centrifuged at 800 × g for 20 min at room temperature. The interphase containing monocytes and lymphocytes was collected and resuspended in PBS. Cells were counted, and then centrifuged again at 300 × g for 10 min. Cell pellets were resuspended in 90 μl MACS buffer and 25 μl anti-CD4 antibodies, anti-CD8a beads, and anti-CD19 beads on ice for 15 min. For negative selection of peripheral blood monocytes, cells were washed, resuspended in MACS buffer, and loaded onto prepared MACS columns. Monocytes were collected from the flow-through washes of the column. Subsequently, viable monocytes (>85% purity) were lysed for determination of CG and β-actin protein. In addition, we also determined CG/NEN protein expression in purified CD11c+ mononuclear phagocyte subsets and neutrophils collected from the lungs of mice challenged with M. bovis BCG, following recently published protocols for the purification of CD11c+ mononuclear phagocytes (15, 23). Neutrophils were purified from lung homogenates of M. bovis BCG-infected mice using a Ly-6G magnetic cell separation (MACS) kit following the instructions of the manufacturer (Miltenyi Biotech).

**Isolation and digestion of lung dLN**

To analyze numbers of DCs and the proliferation of lymphocyte subsets in dLN under various experimental conditions, we also collected dLN of the lung in a 1 ml digestion solution, followed by disruption of the tissue with forceps. Subsequently, dLN were incubated at 37°C in a water bath for 15 min with repeated shaking. A 1-ml digestion solution was added, and lymph node cells were incubated for 15 min. Thereafter, 12 ml FACS buffer was added, and the samples were centrifuged at 400 × g, 4°C, for 5 min. The supernatant was discarded, and the pellet was resuspended in 2 ml FACS buffer and filtered through a 40-μm cell strainer. Cells were counted, followed by FACS analysis of dLN cellular constituents.

**Immunophenotypic analysis of mononuclear phagocyte and lymphocyte subsets in lung parenchymal tissue and dLN**

CD11c+ mononuclear phagocyte subsets as well as lymphocyte subsets isolated from lung parenchyma and lung dLN were immunophenotypically analyzed according to their cell surface Ag expression profiles, as described previously (24, 25). First, the respective CD11c+ mononuclear phagocyte subsets purified from lung homogenates of CG/NEN-deficient and wild-type mice were gated according to their forward light scatter (FSC)-A versus side light scatter (SSC)-A characteristics and FSC-A versus autofluorescence profile. Lymphoid/mononuclear cells were further characterized as conventional DCs (CD11b-/+CD11c+/-/MHCII-/+), or as highly autofluorescent lung macrophages (F4/80+/CD11b+CD11c-/-/MHCII-/+), or as autofluorescent exudate macrophages (F4/80+/CD11b+CD11c+/-/MHCII-/+). Immunophenotypic analysis of lymphocyte subsets in lung dLN was done by gating according to their FSC-A versus SSC-A characteristics, followed by characterization of CD4+ T cells (CD3+/CD4+/-CD8+/-), CD8+ T cells (CD3+/CD4-/-CD8+), and B cells (CD3-/CD19+).****

**Isolation of alveolar macrophages and treatment with liposomal CG and NE**

Alveolar macrophages were recovered from untreated mice by BAL, and 1 million BAL cells (>99% alveolar macrophages) were seeded into cell culture dishes in RPMI 1640 supplemented with 10% FCS, 1% glutamine, 1% penicillin, and streptomycin. Alveolar macrophages were allowed to adhere to the plastic surface for 1 h. Then, cells were washed three times with cell culture media without FCS to remove nonadherent cells, and 100 μl liposomal CG/NEN or empty liposomes as control were added. After 3 and 6 h of liposomal phagocytosis, alveolar macrophages were lysed by Western blot analysis of immunoreactive CG/NEN.

**Generation of chimeric wild-type mice**

To better dissect the roles of CG/NEN activity of resident lung cells versus circulating monocyte subsets to contribute to mycobacterial killing, we subjected wild-type mice to whole body irradiation, and then transplanted these mice with bone marrow collected from CG/NEN double-mutant mice to generate chimeric wild-type mice with a CG/NEN-deficient hematopoietic system. For isolation of bone marrow cells, tibias and femurs of sex-matched CG/NEN KO and wild-type donor mice were flushed with RPMI 1640 containing 10% FCS, and single-cell suspensions were prepared. Cell aggregates were removed by careful filtration of the cell suspension through 40-μm nylon meshes. Bone marrow cell suspensions were washed twice in Leibovitz L15 medium. Recipient wild-type mice received a total body irradiation of 8 Gy delivered by a linear accelerator (21). Within 16 h after irradiation, recipient wild-type mice received ~10^7 CG/NEN KO bone marrow cells or wild-type bone marrow cells (transplant controls) suspended in Leibovitz medium without supplements via lateral tail vein injections. Resulting chimeric wild-type mice exhibiting a CG/NEN-deficient hematopoietic system, but wild-type alveolar and lung parenchymal macrophages, were housed under specific pathogen-free conditions with free access to autoclaved food and water for 5 wk, before M. bovis BCG infections were initiated.

**Western blot analysis**

For cell-specific detection of CG and NE protein in BAL cells or monocytes or CD11c+ lung mononuclear phagocytes or neutrophils, purified cell populations were lysed in ice-cold lysis buffer (100 μl/1×10^6 cells), and total protein concentrations of lysates were determined by BCA Pierce assay (Thermo Scientific, Waltham, MA), according to the manufacturer’s instructions (26). For SDS-PAGE, protein samples diluted 1:4 with 4× reducing Roti-Load buffer (Roth, Karlsruhe, Germany) were heated for 15 min at 99°C, and then cooled at room temperature prior to loading of equal amount of protein (15 μg) and Precision Plus Protein Standard (Dual Color; Bio-Rad, München, Germany) onto a 12.5% SDS-polyacrylamide gel. Separated proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA) via semidry blotting. Detection of CG/NEN immunoreactivity was done using an anti-CG/NE clone I-19 (Santa Cruz Biotechnology) with specificity for CG and NE cross-reacting with NE (K. Steinwede and U. Maass, unpublished observations) using chemiluminescence ECL substrates (GE Healthcare, Buckinghamshire, U.K.). Stripped blots were reprobed with anti-β-actin mAb to verify equal sample loading.

**Measurement of liposomal encapsulated neutrophil serine protease content using fluorescence resonance energy transfer substrates**

Amounts of liposomal encapsulated CG and NE were determined, as described recently (10, 27, 28). Briefly, 100 μl CG/NEN-loaded liposomes were centrifuged at 13,000 × rpm at 4°C for 30 min. Subsequently, liposome pellets were resuspended in 200 μl ice-cold PBS supplemented with 0.15% Brij 35 (Sigma-Aldrich, Steinheim, Germany), followed by one freeze/thaw cycle. Then, liposome preparations were sonicated for 3 × 10 s with 1-min break on ice (UW 2200; Bandelin Electronics, Berlin, Germany). Liposome lysates were centrifuged at 13,000 rpm at 4°C for 25 min, resulting in supernatants and pellets, respectively. Supernatants and pellets of lysed CG/NEN liposomes were subjected to kinetic measurements of peptidase activities to determine their CG/NEN content using highly sensitive Abz-peptidyl-EDDnp fluorescence resonance energy transfer (FRET) substrates, which fully discriminate between these two neutrophil serine proteases, as described recently (10, 27, 28).

**Lung histopathology**

Histopathological examination of formalin-fixed, paraffin-embedded, and H&E-stained lung tissue sections (3 μm) from wild-type mice, CG KO mice, and CG/NEN double-KO mice infected with M. bovis BCG was done under blinded conditions by an experienced lung pathologist (F.L.) using a Zeiss Axiosvert 200 M microscope (29).

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Confocal laser-scanning microscopy

Confocal laser-scanning microscopy was performed using an inverted LSM 510 META microscope (Zeiss, Jena, Germany) equipped with a C-Apochromat 40×/1.2 W corr water immersion objective, and a helium-neon laser (543 nm excitation). GFP (green) and PKH26-GL (red) emissions were detected with 505/530 and 560/615 band pass filters, respectively. The transmitted light photomultiplier was used for bright field images of samples. Images were analyzed using Imaris (Bitplane) software.

Electron microscopy

Alveolar macrophage suspensions were centrifuged at 10,000 rpm and fixed by immersion in fixation solution composed of 1.5% glutaraldehyde (Agar Scientific, Essex, U.K.) and 1.5% formaldehyde freshly prepared from depolymerized paraformaldehyde (Merck Chemicals, Darmstadt, Germany) in 0.15 M HEPES buffer (Sigma-Aldrich, Hamburg, Germany; total osmolarity of 800 mosmol/L and a vehicle osmolarity of 300 mosm/kg at pH 7.35) for at least 3 h. The suspensions were centrifuged at 10,000 rpm for 5 min after each of the following processing steps. After repeated rinsing in 0.15 M HEPES buffer and in 0.1 mmol/L cacodylate buffer (Plano, Wetzlar, Germany), stabilization of predominantly unsaturated lipids was achieved by postfixation with 1% osmium tetroxide (Plano, Wetzlar, Germany) in 0.1 M cacodylate buffer, followed by rinsing in 0.1 M cacodylate buffer (2 × 5 min). After twice rinsing in distilled water, specimens were stained en bloc overnight (12–18 h) with a mixture of equal portions of uranyl-acetate and water (half-saturated aqueous uranyl acetate solution, 1:1; Agar Scientific, Stansted, Essex, U.K.) at 4–8°C. Cell suspensions were dehydrated in an ascending series of acetone (70, 90, and 100%) (J. T. Baker, Deventer, The Netherlands) and embedded in epon (SERVA Feinbiochemica, Heidelberg, Germany). Ultrathin sections (70 nm) were cut by an ultra microtome (Reichert Ultracut S, München, Germany), collected on nickel grids (Plano), and stained with lead citrate (Merck, Darmstadt, Germany) and uranyl acetate. Ultrathin sections were examined with an electron microscope (Morgagni 268; FEL, Eindhoven, The Netherlands). Electron micrographs of representative areas were taken by a digital camera (Veleta TEM camera; Olympus Europe Holding, Hamburg, Germany).

ELISA

TNF-α and CCL2 release in BAL fluids of uninfected or M. bovis BCG-infected CG-deficient, CG/NE double-mutant, or wild-type mice was determined using commercially available ELISA, according to the manufacturer’s instructions (R&D Systems).

Statistics

All data are given as mean ± SEM. Differences between controls and respective treatment groups over time were analyzed by ANOVA, followed by post hoc Dunnett test. Significant differences between groups were analyzed by Levene’s test for equality of variances, followed by Student t test using SPSS for Windows software package. Statistically significant differences between various treatment groups were assumed when p values were <0.05.

Results

Effect of CG deficiency on pathogen elimination in M. bovis BCG-infected mice

Our recently made observation that CG, but not cathepsin B, K, L, D, or S, mRNA is strongly upregulated in sorted alveolar macrophages of mice challenged with M. bovis BCG in vivo (15) prompted us to analyze whether selective deletion of CG would influence protective immunity in mice in response to M. bovis BCG infection. As shown in Fig. 1, CG KO mice infected with M. bovis BCG showed significantly increased mycobacterial loads in their BAL cells on day 28 postinfection and, even more pronounced, in lung parenchymal tissue on days 14 and 28 postinfection (Fig. 1A, 1B). CG deficiency had no significant impact on dissemination of M. bovis BCG into extrapulmonary organ systems such as the spleen and lung dLN during an observation period of 28 d (Fig. 1C, 1D).

Effect of CG and NE deficiency on pulmonary protective immunity against M. bovis BCG

Because CG is part of a serine protease network, the members of which are all stored in similar amounts in neutrophil azurophil

![FIGURE 1. Determination of mycobacterial loads in the lung, spleen, and lung dLN of CG-deficient and wild-type mice infected with M. bovis BCG. Wild-type mice (black bars) and CG-deficient mice (white bars) were infected with M. bovis BCG (2 × 10⁵ CFU/mouse), and mycobacterial loads were determined in BAL cells (A), lung tissue (B), spleen (C), and lung dLN (D). Values are shown as mean ± SEM for n = 9 mice per time point and treatment group. **p < 0.03, ***p < 0.001, significant difference relative to wild-type mice.]
granules, we addressed the question whether additional KO of NE, a serine protease with strong homology to CG, would further attenuate pulmonary resistance of double-mutant mice to challenge with *M. bovis* BCG. Relative to wild-type mice, mice deficient of both serine proteases CG and NE demonstrated significantly increased mycobacterial loads both in BAL cells and lung parenchymal tissue as early as 14 d postinfection with *M. bovis* BCG (Fig. 2A, 2B), which were also significantly increased when compared with CG KO mice at this time point (compare with Fig. 1), thus demonstrating that additional knockout of NE further attenuated pulmonary resistance to mycobacterial infection in mice. Moreover, significantly increased bacterial loads were also noted in the spleens of CG/NE-deficient mice relative to wild-type mice by day 28 postinfection with *M. bovis* BCG (Fig. 2C). In contrast, no differences in mycobacterial loads were observed in dLN between groups (Fig. 2D). Histopathological examination of lung tissue sections collected from *M. bovis* BCG-infected wild-type mice, CG KO mice, and CG/NE double-KO mice revealed that, particularly on day 28 postinfection, lung granuloma formation was significantly increased in CG KO mice and even further increased in CG/NE KO mice, relative to wild-type mice (Fig. 3A, 3B), whereas on day 56 postinfection, no difference in the degree of lung granuloma formation was noted between groups (data not shown).

In addition, significantly increased TNF-α protein levels in BAL fluids were observed in CG/NE KO, but not CG KO mice after 28 d postinfection (Fig. 3C), indicating an increased proinflammatory response in these mice.

**FIGURE 2.** Determination of mycobacterial loads in the lung, spleen, and lung dLN of CG/NE-deficient and wild-type mice infected with *M. bovis* BCG. Wild-type and CG/NE-deficient mice were infected with *M. bovis* BCG (2 × 10⁵ CFU/mouse). At the indicated time points, mycobacterial loads were determined in BAL cells (A), lung tissue (B), spleen (C), and dLN (D) of wild-type mice (black bars) and CG/NE-deficient mice (white bars). Values are shown as mean ± SEM for *n* = 9 mice per time point and treatment group. **p < 0.03, ***p < 0.001, significant difference relative to wild-type mice.

**FIGURE 3.** Effect of CG/NE deficiency on lung-protective immunity against *M. bovis* BCG. Wild-type, CG-deficient, and CG/NE-deficient mice were infected with *M. bovis* BCG (2 × 10⁵ CFU/mouse). At indicated time points, whole lungs were removed, and lung tissue sections were prepared for histopathologic examination (HE stain) (A) and lung morphometric examination (B). Representative photographs are original magnification ×2.5 (A). (C) TNF-α levels in BAL fluids of wild-type (black bars), CG-deficient (white bars), and CG/NE-deficient mice (gray bars) postinfection with *M. bovis* BCG. Values are shown as mean ± SEM for *n* = 9 mice per time point and treatment group. *p < 0.05, ***p < 0.001, significant difference relative to wild-type mice.

Leukocyte subset recruitment profiles in wild-type mice, CG-deficient mice, and CG/NE double-mutant mice infected with *M. bovis* BCG

Previous reports have suggested a role for CG in leukocyte extravasation (6, 30, 31). The described differences between wild-type mice, CG KO, and CG/NE KO mice in terms of mycobacterial pathogen elimination and induction of lung granuloma formation might thus be explained by differences in lung leukocyte subset recruitment in response to mycobacterial infection. Therefore, we analyzed leukocyte subset profiles in BAL fluids of mice of the various treatment groups postinfection with *M. bovis* BCG. As shown in Supplemental Fig. 1, no significant differences in
numbers of exudate macrophages, neutrophils, or lymphocytes were observed in BAL fluids of CG-deficient, CG/NE-deficient, or wild-type mice in response to *M. bovis* BCG infection, with peak alveolar neutrophil and exudate macrophage numbers observed by day 28 postinfection, followed by peak alveolar lymphocyte numbers noted on day 56 postinfection (Supplemental Fig. 1A–D). Moreover, numbers of lung mononuclear phagocyte subsets and CD45<sup>pos</sup>/Gr-1<sup>pos</sup> neutrophils in lung parenchymal tissue were not significantly different between *M. bovis* BCG-infected CG/NE-deficient mice relative to wild-type mice (data not shown). Also, FACS analysis did not reveal any differences in mobilization of conventional lung DC toward lung dLN, or numbers of lymphocyte subsets within lung dLN in response to *M. bovis* BCG infection in CG or CG/NE KO mice when compared with wild-type mice by day 7 up to day 56 postinfection (data not shown). Together, these data demonstrate that neutral serine proteases CG/NE are not required for either leukocyte recruitment into the lungs or lung dLN or numbers of lymphocytes within lung dLN of mice infected with *M. bovis* BCG.

**Effect of purified CG on *M. bovis* BCG growth under aerobic and anaerobic conditions in vitro**

The finding that deficiency of CG significantly decreased the containment of *M. bovis* BCG in the lungs of mice prompted us to analyze whether purified CG would exert direct antimycobacterial effects in vitro. As shown in Fig. 4A, using aerobic culture conditions, incubation of *M. bovis* BCG with purified human CG dose dependently impaired the mycobacterial outgrowth during an observation period of 72 h in vitro, with lowest significantly active CG protein levels amounting to 10 μg/ml culture medium. In contrast, using anaerobic culture conditions, in which metabolic activities of mycobacteria are drastically affected, significantly reduced mycobacterial outgrowth in vitro was only achieved in the presence of CG applied at 50 and 100 μg/ml (Fig. 4B). Importantly, under both aerobic and anaerobic experimental conditions, addition of CtsB as a control protease did not impair mycobacterial growth in vitro (Fig. 4).

**Identification of neutrophils as the main cellular source of CG and NE in mice**

We further aimed at identifying the cellular source mediating CG/NE-dependent innate immune responses in mice after mycobacterial challenge. CG and NE are localized in azurophilic granules of neutrophils (6, 7). Increased CG mRNA levels were also found in alveolar macrophages of wild-type mice infected with *M. bovis* BCG (15), suggesting that both types of professional phagocytes might contribute to CG bioactivity in mice in response to mycobacterial challenge. In initial experiments, BAL fluid cells collected from the lungs of *M. bovis* BCG-infected wild-type mice were subjected to Western blot analysis of CG/NE protein on days 14, 28, and 56 postinfection. As shown in Fig. 5A, both untreated wild-type mice and mice infected for 14 d with *M. bovis* BCG did not show detectable CG/NE protein in their BAL cells, whereas CG/NE protein was easily detectable in whole BAL cell lysates.
collected from mice at 28 and 56 d postinfection, thereby strongly correlating with peak alveolar neutrophil influx observed in the lungs of these mice (Supplemental Fig. 1C). In contrast, no immunoreactivity for CG/NE was detectable in BAL cell lysates of M. bovis BCG-infected CG/NE double-mutant mice (Fig. 5A). Interestingly, neither peripheral blood monocytes nor CD11c+ mononuclear phagocytes (alveolar and lung macrophages and myeloid DC subsets) purified from lung parenchymal tissue of mice infected with M. bovis BCG demonstrated any detectable CG/NE protein expression by Western blot analysis (Fig. 5B, 5C). In striking contrast, lung parenchymal tissue neutrophils collected from mice at day 28 postinfection with M. bovis BCG were found to express CG/NE protein (Fig. 5C). This indicates that recruited neutrophils are the most likely cellular shuttle transporting neutral serine protease bioactivity into the bronchoalveolar compartment of mice in response to infection with M. bovis BCG.

**Hematopoietic CG/NE bioactivity is an important factor in lung-protective immunity against mycobacterial pathogens**

Having demonstrated that alveolar neutrophil recruitment underlies increased CG immunoreactivity in the bronchoalveolar compartment of mice postinfection with M. bovis BCG, we hypothesized that selective hematopoietic deletion of CG/NE bioavailability would attenuate lung-protective immunity against M. bovis BCG. To test this hypothesis, wild-type mice were irradiated and reconstituted with bone marrow cells from wild-type mice (transplant controls) or CG/NE double-KO mice. As expected, transfer of wild-type bone marrow led to strong CG/NE immunoreactivity in BAL fluid cells collected at 24 h post-LPS challenge (Fig. 6A), whereas mice receiving bone marrow from the dual mutants showed >90% reduced CG/NE immunoreactivity in their BAL cells at 24 h posttreatment (Fig. 6A). We found no CG/NE immunoreactivity in BAL fluid cells 28 d postinfection of mutant chimeras with M. bovis BCG (Fig. 6B). Notably, hematopoietic deletion of CG/NE in chimeric wild-type mice provoked

![FIGURE 5. Analysis of CG/NE protein expression in BAL cells, peripheral blood monocytes, CD11c+ mononuclear phagocytes, or neutrophils (PMN) from lung parenchymal tissue of M. bovis BCG-infected mice. Wild-type mice and CG/NE-deficient mice were either mock infected or infected with M. bovis BCG (2 × 10^5 CFU/mouse) for 14, 28, and 56 d. Subsequently, mice were subjected to BAL for isolation of BAL cells (A), or monocytes were purified from peripheral blood of wild-type mice (B), or CD11c+ mononuclear phagocyte subsets and neutrophils were isolated from lung parenchymal tissue of wild-type mice (C) at the indicated time points. Subsequently, CG/NE protein expression was determined by Western blot analysis. Control represents purified human CG (left lane) and purified human NE (right lane). Each Western blot analysis is representative of at least n = 3 independent experiments.](http://www.jimmunol.org/)

![FIGURE 6. Effect of a hematopoietic CG/NE deletion on CG/NE protein content in BAL fluids of LPS-treated or M. bovis BCG-infected mice and mycobacterial pathogen elimination in the lung and spleen of chimeric wild-type mice. Wild-type mice were irradiated, followed by reconstitution of their hematopoietic system with bone marrow cells collected from CG/NE double-mutant mice (chimeric wild type) or with bone marrow cells collected from wild-type mice (transplant control). (A) At 5 wk post-BMT, mice were challenged intratracheally with 20 μg LPS. At 24 h post-LPS treatment, transplant control mice (left lane) and chimeric wild-type mice (right lane) were subjected to BAL for collection of BAL fluid cellular constituents (>95% neutrophils). (B) At 5 wk post-BMT, mice were infected with M. bovis BCG (2 × 10^5 CFU/mouse), and BAL fluid cellular constituents of transplant control mice (left lane) and chimeric wild-type mice (right lane) collected at day 28 postinfection were subjected to analysis of CG/NE immunoreactivity. (C, D) Mycobacterial loads in transplant control mice (black bars) and chimeric wild-type mice (white bars) in BAL cells (C) and lung tissue (D). The given Western blots are representatives of at least three independent determinations. Values are shown as mean ± SEM for n = 9 mice per time point and treatment group. **p < 0.03, significant difference relative to transplant control mice.](http://www.jimmunol.org/)
significantly increased mycobacterial loads in the bronchoalveolar and lung parenchymal compartment of these mice 14 and 28 d postinfection, when compared with M. bovis BCG-infected transplant control mice (Fig. 6C, 6D). In addition, depletion of neutrophils with anti-Ly6G Ab 1A8 applied once daily starting by day 24 after M. bovis BCG infection in wild-type mice resulted in increased lung bacterial loads at day 28 postinfection when compared with control IgG-treated M. bovis BCG-infected wild-type mice (data not shown). Together, these data demonstrate that elicited neutrophils harboring CG/NE contribute to early resistance of the lung to aerogenic mycobacterial infections.

**Therapeutic treatment of mice with liposome-encapsulated CG/NE improves lung innate immunity against M. bovis BCG infection**

The earliest steps in lung mycobacterial infections include invasion of alveolar macrophages by mycobacteria, followed by phagosomal arrest induction in macrophages to prevent phagolysosomal fusion and proteolytic degradation of the pathogen. We asked whether an increased intracellular uptake of serine protease activities by mycobacteria-infected alveolar macrophages would increase their antimycobacterial activities and reduce bacterial loads in distal air spaces during the early phase of infection. To avoid that CG and NE released within the lungs of mice would mediate unspecific lung injury or would be inactivated by endogenous serine protease inhibitors, we encapsulated CG/NE into liposomes, which, according to our previous studies, are very efficiently phagocytosed by alveolar macrophages (18). Successful encapsulation of CG and NE into liposomes was verified by Western blotting, as shown in Fig. 7A. Subsequent FRET analysis of proteolytic activities of CG and NE in lysed liposomal preparations (separated into liposomal pellets and supernatants) revealed the greatest portion of CG enzymatic activity to be located in the pellets of liposomal lysates, whereas NE was primarily found in the supernatant of lysed liposomes (Fig. 7B). This different distribution most probably comes from the strongly cationic character of CG (pI ~ 12) that favors ionic interactions with...
liposomal membrane fragments. Next, incubation of alveolar macrophages with CG/NE-containing liposomes in vitro resulted in increased immunoreactive CG/NE in alveolar macrophages after 3 and 6 h of phagocytosis, whereas no CG/NE protein was detectable in alveolar macrophages incubated with empty PBS-containing liposomes (Fig. 7C), demonstrating that liposome-encapsulated CG/NE indeed accumulates in alveolar macrophages.

To visualize the process of liposomal uptake by alveolar macrophages in vivo, wild-type mice received an intratracheal instillation with FITC-BSA–containing liposomes. Alveolar macrophages collected from mice at 24 h after FITC-BSA liposome instillation demonstrated strong green fluorescence, indicative of liposome uptake (Supplemental Fig. 2). Additionally, we exploited laser-scanning microscopy of alveolar macrophages collected from mice that were previously infected with GFP-expressing M. bovis BCG, followed by therapeutic treatment of mice with CG/NE-loaded liposomes that were stained with the lipophilic red fluorescent dye, PKH26. We were able to show that red fluorescent CG/NE liposomes and GFP-expressing M. bovis BCG colocalized within the same macrophage compartments, as indicated by a yellow overlay fluorescence shown in Fig. 7D. Finally, successful colocalization of mycobacteria with CG/NE liposomes within alveolar macrophages collected from mice previously infected with M. bovis BCG and therapeutically treated with CG/NE-containing liposomes was further confirmed by electron microscopy (Fig. 7E).

We next treated M. bovis BCG-infected wild-type mice therapeutically with a single dose of liposomal CG/NE on day 1 after mycobacterial infection (Fig. 8A). This resulted in significantly decreased mycobacterial loads in the bronchoalveolar and lung

![FIGURE 8](http://www.jimmunol.org/)  
Therapeutic treatment of M. bovis BCG-infected mice with liposomal CG and NE. Wild-type mice were infected with M. bovis BCG (2 \times 10^5 CFU/mouse) and were then therapeutically treated with a single application of CG/NE-containing liposomes or PBS liposomes at day 1 postinfection (A) or a repetitive application of CG/NE-containing liposomes or PBS liposomes at day 1 and 7 postinfection (D). Mycobacterial loads were determined in BAL cells (B, E) and lung tissue (C, F) at the indicated time points. (G, H) Numbers of alveolar macrophages and lymphocytes in BAL fluids of M. bovis BCG-infected mice therapeutically treated with repetitive applications of CG/NE liposomes or PBS liposomes, as indicated. Values are shown as mean ± SEM for n = 6 mice per time point and treatment group. *p < 0.05, **p < 0.03, ***p < 0.001, significant difference relative to wild-type mice treated with PBS liposomes.
Discussion

This study was aimed at elucidating the role of the two neutrophil serine proteases CG and NE in lung-protective immunity against mycobacterial infections induced by *M. bovis* BCG in mice. We found evidence for a critical contribution of the two closely related neutrophil serine proteases, CG and NE, in early resistance to mycobacterial infection. CG mutant mice and, even more pronounced, CG/NE double-mutant mice responded with significantly increased mycobacterial loads both in the bronchoalveolar and parenchymal compartment of mice after 3 and 7 d up until 10 d postinfection (Fig. 8B, 8C). As on day 10 postinfection, we found mycobacterial loads to again increase in mice treated once with liposomal CG/NE therapeutically (Fig. 8B, 8C); we next explored a repetitive liposomal CG/NE therapy in mice infected with *M. bovis* BCG (Fig. 8D). Repeated application of liposomal CG/NE on day 1 and 7 postinfection led to further significantly reduced mycobacterial loads within distal airspaces of mice particularly on day 10 postinfection (Fig. 8E, 8F), with improved antibacterial effects still observed on day 14 postinfection. At the same time, repetitive liposomal CG/NE application into the lungs of *M. bovis* BCG-infected mice triggered increased numbers of alveolar macrophages and lymphocytes on day 10 and 14 postinfection, respectively (Fig. 8G, 8H), whereas recruitment of neutrophils and inflammatory monocytes was not affected (data not shown). Such increased numbers of alveolar macrophages were accompanied by elevated CCL2 levels in BAL fluids in mice receiving repetitive CG/NE liposome therapy (PBS versus CG/NE liposome treated: 18 ± 9 versus 164 ± 116 pg/ml CCL2, day 10 postinfection). Moreover, repeated application of liposomal CG/NE led to increased accumulation of DCs and significantly increased numbers of CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells in lung dLN on day 10 postinfection (Fig. 9). Collectively, the data support our concept that therapeutic application of liposomal CG/NE is an effective novel therapeutic approach to diminish mycobacterial loads within distal airspaces of mice. Therapeutic efficacy wanes after ~4 (to 7) days, suggesting that longer-lasting anti-mycobacterial effects would require repetitive intrapulmonary applications of liposomal encapsulated proteases.

![FIGURE 9. Numbers of DC and T lymphocyte subsets in lung dLN after therapeutic treatment of *M. bovis* BCG-infected mice with liposomal CG and NE. Wild-type mice were infected with *M. bovis* BCG (2 × 10<sup>6</sup> CFU/mouse) and then received therapeutic applications of CG/NE-containing liposomes or PBS-containing liposomes at days 1 and 7 postinfection. At indicated time points, lung dLN were removed and processed for quantification of DC (A), CD4<sup>+</sup> T cells (B), and CD8<sup>+</sup> T cells (C) by flow cytometry. Values are shown as mean ± SEM for *n* = 8 mice per time point and treatment group and *n* = 3 mice for untreated control. *p < 0.05, significant difference relative to wild-type mice treated with PBS-containing liposomes.](http://www.jimmunol.org/)

NE could not be detected by histochemistry in alveolar macrophages (33). As such, we cannot fully exclude that very low amounts of CG and/or NE protein are also present in alveolar macrophages in mice, yet were not detectable with the currently employed Western blotting techniques. However, when considering the results obtained from chimeric wild-type mice lacking CG and NE bioactivity in their hematopoietic system, but not in their lungs, it appears very unlikely that possible trace amounts of CG and/or NE protein derived from alveolar macrophages would exert any major impact on lung-protective immunity against mycobacterial infections.

Two aspects support our concept that increased CG/NE activity observed in the lungs of mice in response to mycobacterial challenge is primarily derived from immigrating blood neutrophils. First, detection of CG and also NE in the lung coincides with peak neutrophil influx 28 d after mycobacterial infection, and CG and NE protein expression was directly detected in purified neutrophils collected from the lungs of *M. bovis* BCG-infected mice. Second, chimeric mice lacking CG and NE in their hematopoietic system recruited similar numbers of neutrophils into their lungs upon mycobacterial infection, but no CG/NE antimicrobial activity was detected in their bronchoalveolar compartment and significantly increased mycobacterial loads both in the bronchoalveolar and parenchymal compartment of mice after 3 and 7 d up until 10 d postinfection (Fig. 8B, 8C). As on day 10 postinfection, we found mycobacterial loads to again increase in mice treated once with liposomal CG/NE therapeutically (Fig. 8B, 8C); we next explored a repetitive liposomal CG/NE therapy in mice infected with *M. bovis* BCG (Fig. 8D). Repeated application of liposomal CG/NE on day 1 and 7 postinfection led to further significantly reduced mycobacterial loads within distal airspaces of mice particularly on day 10 postinfection (Fig. 8E, 8F), with improved antibacterial effects still observed on day 14 postinfection. At the same time, repetitive liposomal CG/NE application into the lungs of *M. bovis* BCG-infected mice triggered increased numbers of alveolar macrophages and lymphocytes on day 10 and 14 postinfection, respectively (Fig. 8G, 8H), whereas recruitment of neutrophils and inflammatory monocytes was not affected (data not shown). Such increased numbers of alveolar macrophages were accompanied by elevated CCL2 levels in BAL fluids in mice receiving repetitive CG/NE liposome therapy (PBS versus CG/NE liposome treated: 18 ± 9 versus 164 ± 116 pg/ml CCL2, day 10 postinfection). Moreover, repeated application of liposomal CG/NE led to increased accumulation of DCs and significantly increased numbers of CD4<sup>+</sup> T cells, but not CD8<sup>+</sup> T cells in lung dLN on day 10 postinfection (Fig. 9). Collectively, the data support our concept that therapeutic application of liposomal CG/NE is an effective novel therapeutic approach to diminish mycobacterial loads within distal airspaces of mice. Therapeutic efficacy wanes after ~4 (to 7) days, suggesting that longer-lasting anti-mycobacterial effects would require repetitive intrapulmonary applications of liposomal encapsulated proteases.

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increased mycobacterial loads were observed. Some residual immunoreactive CG/NE protein detected in chimeric mice receiving mutant bone marrow is most probably due to incomplete eradication of host hematopoiesis (21). In addition, we recently demonstrated in a S. pneumoniae infection model that depletion of circulating neutrophils abrogated immunoreactive CG and NE protein expression in BAL fluids of infected mice (10).

The early phase of mycobacterial infections in mouse lungs is characterized by exponential growth of the mycobacteria within distal lung airspaces (2). Recently, it has been shown that virulent M. tuberculosis is able to turn the apoptotic process of infected macrophages into a necrotic pathway by blocking the formation of an apoptotic envelope, thereby causing bacterial escape and mycobacterial spread throughout the lung (34). During this early phase of infection, extracellular located mycobacteria may be attacked directly by newly recruited neutrophils carrying a substantial CG/NE cargo into the bronchoalveolar space. This scenario is strongly supported by the data of the current study. However, during later phases of mycobacterial infection, most of the mycobacteria are considered to reside within the arrested phagosomal compartment of macrophages and DCs, partly within granulomas (15). Therefore, CG and NE may also contribute to the killing of mycobacteria, once the pathogen has been engulfed by macrophages. One concept supports the idea that the proteolytic activity of phagocytosed apoptotic neutrophils is shuttled into mycobacteria-infected macrophages, thereby possibly supporting the antimycobacterial activity of alveolar macrophages. In line with this concept, Tan et al. (35) recently showed that alveolar macrophages killed M. tuberculosis more efficiently after uptake of apoptotic neutrophils, and, in that report, neutrophil granules were found to colocalize with M. tuberculosis in macrophages, whereas the molecular mechanism of increased M. tuberculosis killing in macrophages was not addressed. Although future experiments are required to further specify a role for apoptotic neutrophil-derived CG/NE-containing granules in mycobacterial killing by infected macrophages, the aforementioned scenario, taken together with the data of the current study, strongly supports the concept that neutrophil-derived CG and NE participate in the early pathogen elimination process developing during the acute phase of this chronic infection. This concept is further supported by the current study showing colocalization of GFP-expressing M. bovis BCG with red fluorescent CG/NE-containing liposomes within alveolar macrophages of infected mice subjected to CG/NE therapy.

The results of the current study support the notion that neutrophils serve a protective role in lung host defense against mycobacterial infections. Recently published reports also suggested either direct or immunomodulatory roles for neutrophils in mycobacterial pathogen elimination (36–41). In tuberculosis, a strict regulation of protective immune responses to mycobacterial challenge is important to limit excessive inflammatory responses, and hence, irreversible loss of lung structure and function (42). Seiler et al. (38) showed that neutrophils regulate early granuloma formation via CXCR3 signaling. Moreover, TNF-α is important in granuloma formation and maintenance (43). Both CG and NE have been described to degrade mature TNF-α (44), thereby limiting neutrophil activation and possibly regulating the overall inflammatory process, including the formation and maintenance of granulomas. In line with this observation, CG- and NE-deficient mice showed increased TNF-α protein levels in their lungs in response to mycobacterial infections, and granuloma formations were found to develop earlier in the lungs of mycobacteria-infected CG/NE KO as compared with CG KO and wild-type mice, despite the fact that similar numbers of alveolar recruited neutrophils were observed in the various treatment groups. Additionally, Reece et al. (16) demonstrated that serine protease activity of CG functions as a protective mechanism within hypoxic lung granulomas during M. tuberculosis infection. Thus, in addition to direct antimicrobial activities of CG and NE, immunomodulatory activities of the examined serine proteases may have contributed to the improved immune response against mycobacterial infections of wild-type mice as opposed to CG KO and CG/NE KO mice.

To the best of our knowledge, the current study for the first time directly investigated the role of neutrophil serine proteases on the lung host defense against mycobacterial infections in mice. The data show that antimicrobial activities of CG and NE particularly contribute to control early mycobacterial outgrowth in the acute phase of mycobacterial infection. Repetitive therapeutic application of liposomal encapsulated human CG/NE significantly reduced mycobacterial loads within the lung. Notably, we observed that >95% of alveolar macrophages participated in uptake of liposomal CG/NE, as judged by flow cytometry and confocal laser-scanning microscopy. We believe this rapid phagocytosis of CG/NE liposomes by macrophages is the major advantage of the chosen liposomal CG/NE-based therapeutic approach, because alveolar macrophages at the same time represent the primary target cell of mycobacterial infection of the lung. As such, this therapeutic approach is cell specific, whereby the encapsulated components are immediately released in target cells within close vicinity to the invading pathogen. Moreover, given that CG and NE are both neutral serine proteases exhibiting their maximal proteolytic activity at neutral pH values, inhibition of macrophage acidification of the phagosomal compartment by engulfed mycobacteria most probably would favor the proteolytic activity of these two proteases in vivo. The liposomal encapsulation of the proteases is an important aspect in safety considerations for the treatment of mycobacteria-infected individuals with liposomal CG/NE to avoid induction of acute lung injury by uncontrolled release of the proteases in the bronchoalveolar compartment, and also to achieve a macrophage-targeting therapeutic approach. Of note, intratracheal application of CG without encapsulation led to significant hemorrhage in mice without any effect on lung mycobacterial loads (data not shown).

Collectively, to our knowledge, we, in this study, for the first time provide evidence that neutral serine proteases CG and the closely related NE critically contribute to resistance against mycobacterial infections in mice. We further successfully established a novel liposome-based therapeutic approach to control mycobacterial outgrowth in the lungs of mice during the early phase of infection in vivo. This strategy bears promise as an adjuvant treatment of life-threatening multiresistant or extensively drug-resistant mycobacterial infections in humans.

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Disclosures
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