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Cathepsin G in Experimental Tuberculosis: Relevance for Antibacterial Protection and Potential for Immunotherapy

Kerstin Walter,*1 Kathrin Steinwede,1 Sahar Aly,§ Thomas Reinheckel,¶ Jennifer Bohling,‡ Ulrich A. Maus,‡2 and Stefan Ehlers*,†,2

Neutrophil serine proteases, such as cathepsin G (CG) and neutrophil elastase (NE), have been implicated in the protective response against infections, including experimental mycobacterial infections. The goal of this study was to explore the role of CG in immunocompetent mice challenged aerogenically with Mycobacterium tuberculosis. We used genetically CG- or CG/NE-deficient mice to define the importance of these neutrophil serine proteases for antibacterial protection, granulomatous response, and survival. In addition, we explored the effect of intratracheally delivered liposomally encapsulated CG/NE as a therapeutic approach early during M. tuberculosis infection. Our data show that the presence of CG or CG/NE prolongs survival in M. tuberculosis–infected mice. However, CG is not directly involved in antibacterial defenses, and exogenous intratracheal administration of CG combined with NE does not reduce bacterial loads in the lungs of M. tuberculosis–infected mice. The Journal of Immunology, 2015, 195: 000–000.

Tuberculosis is the cause of significant morbidity and mortality worldwide (1). The situation is particularly aggravated by HIV coinfection and the emergence of multidrug-resistant strains of the causative organism, Mycobacterium tuberculosis. Therefore, in addition to antibiotic therapy, adjuvant immunologic modification of the immune response to infection appears to be an attractive strategy that warrants further investigation of potential target cells and molecules (2).

The mouse model of tuberculosis has been instrumental in uncovering the relative contribution of macrophages, T cells, and cytokines in the inflammatory and protective antibacterial responses of the host (3, 4). The initial permissive habitat for M. tuberculosis following inhalation into the lung is provided by alveolar macrophages. Over the course of infection, recruited inflammatory, T cell– and IFN-γ–activated macrophages are capable of arresting M. tuberculosis growth in the lung and the spreading of M. tuberculosis into other organs or the environment (5). Growth arrest and killing of M. tuberculosis is the result of a number of bacteriostatic and bacteriocidal mechanisms provided by the activated macrophage, oxidative, and nitrosative pathways being the most prominent, whereas other cells of the myelomonocytic lineage, such as neutrophils and NK cells, and a number of lysosomal effector molecules still await definition of their relative contribution (6). Interestingly, it has been shown that macrophages eliminated M. tuberculosis more efficiently after phagocytosis of apoptotic neutrophils. Because neutrophil granules were found to colocalize with M. tuberculosis in macrophages, it appears possible that neutrophil-derived bacteriocidal molecules play a role in early antibacterial defenses against M. tuberculosis infection (7).

Neutrophil serine proteases, such as cathepsin G (CG) and neutrophil elastase (NE), were implicated in the protective response against streptococcal infections and Gram-negative sepsis (8, 9). Recently, their contribution to the host’s defensive armamentarium against mycobacterial infections was documented in a mouse model of Mycobacterium bovis bacillus Calmette–Guérin (BCG) infection. In this model, a deficiency in CG and—in a more pronounced fashion—in both CG and NE was associated with impaired elimination of BCG. In addition, therapeutically administrated liposomally encapsulated human CG and NE improved lung-protective immunity against BCG (10). In addition, in another model of severely immunocompromised NO synthase 2–deficient mice infected intradermally with M. tuberculosis, CG activity was shown to contribute to containment of M. tuberculosis in hypoxic lung granulomas (11). Thus, CG appears to be an attractive candidate for immune-enhancing therapy for M. tuberculosis infection.

The goal of this study was to explore the role of CG in immunocompetent mice challenged aerogenically with M. tuberculosis. Our approach was 2-fold: 1) the use of genetically CG or CG/NE–deficient mice to define the importance of these neutrophil serine proteases for antibacterial protection, granulomatous response and survival; and 2) therapeutic application of liposomally encapsulated CG/NE early during M. tuberculosis infection to explore the effect of CG/NE supplementation.

Our data show that the presence of CG or CG/NE prolongs survival in M. tuberculosis–infected mice. However, CG is not directly involved in antibacterial defenses, and exogenous administration of CG combined with NE does not reduce bacterial loads in the lungs of M. tuberculosis–infected mice.

Abbreviations used in this article: BCG, bacillus Calmette–Guérin; CG, cathepsin G; KO, knockout; NE, neutrophil elastase; TF, tissue factor; TFPI, tissue factor pathway inhibitor.

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Materials and Methods

Mouse strains

CG- and CG/NE-deficient mice were generated as described in (12) and were provided by Jürgen Röes (University College London Medical School, London, U.K.). Generation of Cathepsin K−/− mice is described previously (13), generation of Cathepsin K−/−/L−/− mice is described in (14, 15), and generation of CathepsinB mice is described by Halangk et al. (16); these cathepsin-deficient mice were provided by Paul Saftig (Christian-Albrechts-University of Kiel, Kiel, Germany). Cathepsin Z-deficient mice were generated as described previously (17). Age- and sex-matched specific pathogen-free wild-type (129 S2/SvPasCrl or C57BL/6) and respective knockout (KO) mice were maintained in individually ventilated cages (Ebeco, Castrop-Rauxel, Germany) under biosafety level III conditions.

Bacteria

*M. tuberculosis* H37Rv was grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with oleic acid, albumin, dextrose, and catalase enrichment medium (Life Technologies, Gaithersburg, MD). 0.05% Tween 80 and 0.2% glycerol (VWR International, Darmstadt, Germany). Mid-exponential phase cultures were harvested, aliquoted, and frozen at −80 °C. After thawing, viable cell counts were determined by plating serial dilutions on Middlebrook 7H10 agar supplemented with 10% bovine serum (BioWest, Nuaille, France) followed by incubation at 37 °C for 3 wk. To ensure proper dispersion of mycobacteria, the bacterial suspension was drawn through a non-pyrogenic needle (Microlac3e, BD, Drogbeda, Ireland) prior to every infection experiment.

Preparation of liposome-encapsulated CG and NE

Liposome encapsulation of CG and NE was done as previously reported (10). Briefly, 4 mg cholesterol was added to 43 mg phosphatidylycholine, 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 two times in sterile PBS and centrifuged again. Subsequently, liposomes were resuspended in 2.5 ml of sterile PBS and kept at 4 °C and used within 24 h after preparation.

Infection of mice

Aerogenic infection with *M. tuberculosis* H37Rv was completed in a Glas-Col aerosol infection device (Glas-Col, Terre-Haute, IN). Inoculum size was confirmed 24 h postinfection by determining the bacterial burden in the lung. To follow the course of infection, bacterial replication in lung, liver, and spleen was evaluated at different time points postinfection. Organs were aseptically removed, weighed and homogenized in distilled sterile water containing 0.05% Tween 80. Tenfold serial dilutions of organ homogenates were plated on Middlebrook 7H10 agar supplemented with 10% bovine serum and incubated at 37 °C for 21 d. Colonies on plates were enumerated, and results were expressed as log10 CFU per organ. Mice that lost 25% of their original weight during the course of infection were scored as moribund and were sacrificed. All animal experimentation was in accordance with the Animal Research Ethics Board of the Ministry of Environment, Nature, Protection and Agriculture (Kiel, Germany).

Intratracheal application of liposome encapsulated CG/NE

Twenty-four hours after *M. tuberculosis* infection, wild-type mice (129 S2/SvPasCrl) were anesthetized with ketamine and xylazine and received repetitive applications of liposomally encapsulated CG/NE (100 μl per mouse) or PBS containing liposomes as control. For intratracheal instillation of liposomes, the trachea was exposed by surgical resection, and a 26-gauge catheter (Abbocath-T; Abbott Ireland, Sligo, Ireland) was inserted into the trachea under stereomicroscopic control. After instillation, wounds were closed with sterile sutures. After the mice fully recovered from anesthesia, they were returned to their cages. Intratracheal instillation was repeated at day 7 and day 14 postinfection. The orotracheal application of liposomes is an alternative method of accessing the lung without direct surgical exposure of the trachea; it permits a higher number of CG/NE-liposome applications at days 1, 3, 7, 10, 14, and 17 after *M. tuberculosis* infection with 100 CFU or CG/NE-liposome application at days 1, 5, 8, and 12 after *M. tuberculosis* infection with 2000 CFU. For this procedure, anesthetized mice were suspended at an ∼45° angle by fixing the two front upper teeth to a metal filament that is attached to a plastic support (Trachealintubation; Föhr Medical Instruments, Seeheim Germany). A small cylinder was placed under the neck to position the larynx, pharynx, and trachea on a straight line. A metal, custom-made laryngoscope and small forceps were used to keep the mouth open and to displace the tongue. The trachea was visualized by percutaneous transillumination using a 150-W halogen light source with flexible fiberoptic arms (Schott Lighting and Imaging, Mainz, Germany) positioned at the surface of the skin just below the vocal cords. In this manner, the vocal cords and the tracheal lumen are clearly visible, and the tracheal lumen appears as a luminous point. During this direct visualization, a 26-gauge catheter was intubated into the trachea to a point at which its proximal end was at level with the upper teeth. One hundred microliters of the liposomal suspension were instilled into the catheter with a syringe. Proper placement of the tube was verified by surveying the oscillation of the liposomal suspension within the catheter that was in synchrony with the movement of the mouse’s thorax when the tube was inserted correctly into the trachea. After endotracheal administration, mice were placed on heating pads until they fully recovered from the treatment before being returned to their cages.

Quantification of cytokine production

To determine chemokine and cytokine levels ex vivo, lung homogenate and plasma were collected from naive mice and *M. tuberculosis*-infected mice at different time points following infection. The concentrations of IL-1β, IL-17A, IL-12IFN-γ, IL-6, TNF, CCL2, CXC1L, CCL5, and MIP-1α were analyzed by Cytometric Bead Array (CBA), FACS analysis, and the respective FCAPArray software according to the manufacturer’s instructions (BD Biosciences, Heidelberg, Germany).

Histology

For histology, lung lobes were fixed in 4% formalin-PBS, set in paraffin blocks, and sectioned (2–3 μm). Histopathologic analyses were performed using standard protocols for H&E staining.

Plasma tissue factor pathway inhibitor

Tissue factor pathway inhibitor (TFPI) concentrations in mouse blood plasma or serum were determined with an ELISA assay according to the manufacturer’s instructions (USCN Life Science and Technology).

Tail bleeding

Animals were anesthetized with a mixture of ketamine and xylazine and placed in prone position. A distal 5–6-mm segment of the tail was amputated with a sterile scalpel, and the tail was immediately immersed in a 50-ml tube containing prewarmed isotonic saline solution. Each animal was monitored until bleeding ceased, and bleeding time was determined using a stop clock. In moribund mice, bleeding time was monitored for a maximum of 10 min.

Statistical analysis

Quantifiable data are expressed as means of individual determinations and standard deviations using GraphPad prism Software. Log-transformed CFU were analyzed by ANOVA and Bonferroni post hoc test. Bleeding time, TFPI concentrations, organ-to-body weight ratio, and chemokine and cytokine concentrations were analyzed with Kruskal–Wallis with Dunn multiple comparison test. Survival data were analyzed using log-rank Mantel Cox test.

Results

Decreased survival of CG- and CG/NE-deficient mice postinfection with *M. tuberculosis*

The observation that CG and NE deficiency in mice was associated with a higher susceptibility to infection with the vaccine strain *M. bovis* BCG (10) raised the question whether absence of these proteases would also have an effect on long-term survival of mice infected with *M. tuberculosis*, the causative agent of human tuberculosis. Until day 128 postinfection with ∼50 CFU *M. tuberculosis*, wild-type, CG-KO, and CG/NE-KO mice demonstrated a similar survival rate (Fig. 1). Thereafter, protease-deficient mice died at a higher rate than wild-type mice did, resulting in a significantly reduced life span of CG- and CG/NE-deficient mice compared with wild-type mice. Half of infected CG-KO mice succumbed to infection at day 187, and 50% of infected CG/NE-KO mice died by day 189, whereas 50% of wild-type mice
mice survived until day 281. Thus, also during infection with *M. tuberculosis*, the absence of the neutrophil serine proteases CG and NE is associated with a higher susceptibility.

We also investigated the relevance of lysosomal cysteine proteinases such as cathepsin K, cathepsin L, cathepsin B, and cathepsin Z during infection with *M. tuberculosis*. With respect to the survival time, no differences between infected wild-type and infected cathepsin-deficient mice were observed (data not shown), indicating a unique role of the neutrophil serine proteases CG and NE during *M. tuberculosis* infection.

Neutrophil serine protease deficiency has only limited effects on *M. tuberculosis* replication

To investigate whether CG- and CG/NE-deficient mice succumbed earlier to infection because of a higher mycobacterial load, mice were challenged with *M. tuberculosis* H37Rv via aerosol (∼100 CFU/lung), and bacterial loads were determined in lungs, livers, and spleens.

The neutrophil serine proteinases NE and CG are major microbicidal effectors of neutrophils (18). Neutrophils together with monocytes represent the prime innate immune cells that enter the pulmonary site of infection in the initial phase of experimental pulmonary tuberculosis in mice (19). Therefore, we first analyzed the mycobacterial burden during the acute phase of infection (Fig. 2A). In all three mouse strains, the pulmonary replication rate of mycobacteria was of similar magnitude at day 10 and day 20 postinfection. As replication proceeded in the lungs, the bacterial pathogen elimination in *M. tuberculosis*-infected neutrophil serine protease deficient mice in comparison with wild-type mice. Moreover, CG/NE-deficient mice showed decreased pulmonary bacterial replication at day 30 postinfection, and CG and CG/NE deficiency resulted in significantly reduced bacterial loads in spleens of moribund mice, excluding impaired pathogen elimination as a cause for reduced survival rates in these mice.

**Minor histopathologic changes in *M. tuberculosis*-infected neutrophil serine protease–deficient mice**

To assess the formation of granulomas after aerosol infection with *M. tuberculosis*, lung tissue sections from wild-type and CG-KO or CG/NE-KO mice were histologically examined at days 28, 55, and 139 postinfection (Fig. 3). At day 28 postinfection with 200 CFU of *M. tuberculosis*, no differences in the overall degree of lung granuloma formation were observed among the three mouse strains (Fig. 3A). However, whereas 129 S2 mice developed well-demarcated round granulomas the inflammatory foci of neutrophil serine protease–deficient mice were characterized by a stronger perivascular and peribronchiolar lymphocytic cuffing. At day 55 postinfection with 200 CFU of *M. tuberculosis* wild-type, CG- and CG/NE-deficient mice showed loosely organized granulomas with perivascular cuffing and foci of lymphocytes interspersed amid epithelioid macrophages (Fig. 3B). Neutrophil serine protease–deficient mice demonstrated poorly demarcated granulomatus lesions accompanied by alveolar cell wall thickening and interstitial pneumonia. At day 139 postinfection with 100 CFU of *M. tuberculosis*, all three mouse strains showed a pronounced inflammatory cell infiltration that caused a pulmonary consolidation of ∼60% in neutrophil serine protease–deficient and wild-type mice. The degree of necrotic areas was also similar in all three genotypes (Fig. 3C).

The relevance of CG and NE for inflammation was also assessed by monitoring the lung-to-body weight and the spleen-to-body weight ratios (Fig. 4). At the time when neutrophil serine protease–deficient mice became moribund, the lung-to-body weight ratio was significantly increased in CG-KO mice compared with the wild-type mice (Fig. 4A). In addition, CG- and CG/NE-deficient mice developed a splenomegaly with a significantly increased spleen-to-body weight ratio (Fig. 4B). However, histologic examination of the spleen did not reveal any morphologic differences between neutrophil serine protease–deficient mice and wild-type mice with respect to the red and white pulp and the germinal centers (data not shown). The higher organ weights in CG- and CG/NE-deficient mice indicate that neutrophil serine proteases influence the inflammatory response during *M. tuberculosis* infection.

**Altered proinflammatory cytokine and chemokine profiles in lungs and plasma from *M. tuberculosis*-infected neutrophil serine protease deficient mice**

Following activation, neutrophils release their serine proteases to the extracellular environment, where they have an important role in the modulation of the inflammatory response by modifying chemokine activity and activation or inactivation of cytokines (20). Hence, the proinflammatory cytokine and chemokine protein patterns were examined in lungs and plasma of mice challenged with *M. tuberculosis*. In uninfected mice, the protein levels were negligible (Fig. 5A). Upon *M. tuberculosis* infection, pulmonary cytokine and chemokine synthesis increased in wild-type and neutrophil serine protease deficient mice. At day 28 postinfection with 200 CFU of *M. tuberculosis*, wild-type mice expressed significantly higher amounts of the proinflammatory cytokines TNF, IL-1β, and IL-17A than neutrophil serine protease–deficient mice did. In addition, the protein levels of MIP-1α and CCL2 were...
elevated in lungs from wild-type mice when compared with neutrophil serine protease–deficient mice. At day 55 postinfection, wild-type and neutrophil serine protease–deficient mice expressed similar levels of these cytokines and chemokines (Fig. 5A). In striking contrast, when neutrophil serine protease–deficient mice became moribund, pulmonary protein levels of the proinflammatory mediators TNF and IL-6 were significantly increased in CG- and CG/NE-KO mice relative to the wild-type mice (Fig. 5B). A similar pattern was seen for IL-1β. Furthermore, the amount of chemokines, such as MIP-1α, CCL2, and CXCL1, were significantly higher in lung homogenates of neutrophil serine protease–deficient mice compared with wild-type mice. The protein amounts of IL-12/IL-23p40 and IL-17A were similar in wild-type and CG/NE-deficient mice at this late time postinfection (data not shown). Moreover, in plasma of CG- and CG/NE-KO mice, the concentration of TNF was significantly increased relative to the wild-type mice (Fig. 5C). Naive CG/NE-KO, CG-KO, and wild-type mice had similar serum concentrations of these cytokines and chemokines (data not shown).

These data suggest that neutrophil serine proteases are involved in a timely proinflammatory reaction during the early phase of M. tuberculosis infection and, in addition, may prevent an exacerbated inflammatory response during the chronic stage of tuberculosis infection by modulating the expression profile of key proinflammatory cytokines and chemokines.

Prolonged bleeding time but similar TFPI concentration in neutrophil serine protease–deficient mice

Blood neutrophils not only provide the first line of defense against pathogens; they also have a role in blood coagulation (21). To investigate whether neutrophil serine proteases modulate coagulation in experimental tuberculosis, we assessed the tail bleeding time of CG-KO, CG/NE-KO, and wild-type mice before infection, during the chronic stage of infection, and at the time point when neutrophil serine protease–deficient mice became moribund (Fig. 6A). In uninfected mice, the mean bleeding time was prolonged ~2-fold in neutrophil serine protease deficient mice relative to wild-type mice with a mean bleeding time of 104 s for wild-type mice and 245 s and 240 s for CG-KO or CG/NE-KO mice, respectively. At day 77 postinfection, again a significantly 2-fold increased bleeding time (mean bleeding time of 102 s for wild-type mice, 167 s for CG-KO...
mice, and 234 s for CG/NE-KO mice) was observed in neutrophil serine protease–deficient mice. In addition, at the time when neutrophil serine protease deficient mice became moribund, the bleeding time of these mice was again ∼2-fold increased (125 s for wild-type mice, 244 s for CG-KO mice, and 208 s for CG/NE-KO mice). This indicates that during the course of tuberculosis infection, the difference in bleeding time between the mouse strains did not change considerably.

In vivo the prime trigger to blood coagulation is the tissue factor (TF), which is constitutively expressed by perivascular cells and hidden in the intact vessel. In response to vessel wall injury, TF is exposed to the blood, and it can interact with the blood-based factor VIIa to trigger the coagulation cascade. TF-induced coagulation is controlled by the TFPI, which blocks the VIIa/TF complex (22). It was recently demonstrated that neutrophil serine proteases stimulate TF-dependent coagulation by local proteolytic inactivation of the coagulation suppressor TFPI (23). Therefore, we next investigated whether the prolonged bleeding time of neutrophil serine protease–deficient mice was a result of an increased TFPI level. At the time when M. tuberculosis–infected neutrophil serine protease deficient mice became moribund, plasma TFPI levels were analyzed with ELISA. The concentrations of plasma TFPI did not differ among the wild-type, CG-KO, and CG/NE-KO mice (Fig. 6B). Thus, although the hemostatic system of neutrophil serine protease–deficient mice is impaired in its ability to prevent blood loss, there is no difference in plasma TFPI concentration between the three mouse strains.

Treatment of mice with liposome-encapsulated CG/NE does not confer protection during infection with M. tuberculosis

Steinwede et al. (10) demonstrated that therapeutically applied human CG/NE encapsulated in liposomes reduced the mycobacterial loads in lungs of mice infected with the vaccine strain, M. bovis BCG. In this study, we aimed to investigate the effect of these neutrophil serine proteases during infection with M. tuberculosis, the causative agent of human tuberculosis. Human CG/NE was encapsulated into liposomes to prevent nonspecific lung tissue injury or inactivation by endogenous serine protease inhibitors. Fluorescence resonance energy transfer analysis of lysed liposomal preparations confirmed the enzymatic activity of both enzymes (data not shown). Wild-type mice were aerogenically infected with a low dose (Fig. 7A, 7B) or a high dose (Fig. 7C) of M. tuberculosis H37Rv. After intratracheal (Fig. 7A) or orotracheal (Fig. 7B, 7C) administration of CG/NE–loaded liposomes at defined time points
postinfection, their therapeutic potential was evaluated on the basis of pulmonary bacterial replication. In a first experiment, wild-type mice were challenged with a low dose of \textit{M. tuberculosis} followed by intratracheal instillation of PBS or CG/NE liposomes at days 1, 7, and 14 postinfection (Fig. 7A). On days 10 and 21 postinfection, the bacterial burden in the lung tissue was similar irrespective of the treatment.

It appeared possible that during infection with virulent \textit{M. tuberculosis}, additional administrations might be necessary to achieve a therapeutic effect with liposomally encapsulated CG/NE. Therefore, in the following experiment, therapeutic treatment was performed at days 1, 3, 7, 10, 14, and 17 after low-dose aerosol infection (Fig. 7B). At days 13 and 21 postinfection, mice treated with PBS or CG/NE liposomes showed similar bacterial burden in the lung tissue.

In the experiments reported by Steinwede et al. (10), intratracheal infection of mice with a relatively high dose of $2 \times 10^5$ CFU of \textit{M. bovis} BCG followed by instillation of CG/NE–loaded

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Organ-to-body weight ratio of moribund neutrophil serine protease–deficient mice. CG-KO, CG/NE-KO, and wild-type mice were aero
genically infected with $\sim 100$ CFU \textit{M. tuberculosis} and lung (A) or spleen (B) weights and body weights were determined at day 139 postinfection. Data are representative of two independent experiments and shown as mean ± SD of 15–17 mice per group. Significance of organ-to- body weight differences was assessed with Kruskal–Wallis and Dunn test. *p < 0.05, **p < 0.01, ***p < 0.001.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Proinflammatory cytokine and chemokine production in lungs of CG-, CG/NE–deficient, and wild-type mice. Mice were challenged with (A) $\sim 200$ CFU or (B and C) $\sim 100$ CFU of \textit{M. tuberculosis}. Cytokine and chemokine levels in lung homogenates (A and B) and plasma (C) collected at indicated time points following infection were determined by cytometric bead array and shown as mean ± SD of 3–5 mice (A), 15–17 (B), and 14–16 (C) mice per group. Data (A and B) are representative of two independent experiments. Statistical analysis was performed using Kruskal–Wallis and Dunn test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
liposomes resulted in a colocalization of mycobacteria and liposomes within the same compartment of alveolar macrophages. To increase the likelihood of such a colocalization in the next experimental setting, mice were aerogenically infected with a high dose of \(M. tuberculosis\) followed by orotracheal instillation of liposomes at days 1, 5, 8, and 12 postinfection (Fig. 7C). At day 14 postinfection, the pulmonary bacterial burden was similar in mice treated either with PBS- or CG/NE–loaded liposomes. Taken together, these results clearly demonstrate that repetitive instillation of liposomally encapsulated CG/NE does not contribute to a reduction of \(M. tuberculosis\) replication during the early phase of antimycobacterial response, regardless of the number of instillations applied or the dose of infection used.

Discussion
Cathepsins have been reasoned to contribute to protection against \(M. tuberculosis\) infection in various ways. Cathepsin K was initially promoted as a marker of macrophage differentiation and was reported to be highly expressed in epithelioid cells and multinucleated giant cells during mycobacterial infection (24). It was shown to be a sensitive immunohistochemical criterion for the detection of microgranulomas in hypersensitivity pneumonitis (25). Similarly, cathepsin B accumulates in delayed-type hypersensitivity reactions induced by \(M. tuberculosis\) (26). Cathepsin L maturation and activity is impaired in macrophages harboring \(M. tuberculosis\), indicating that \(M. tuberculosis\) modulates the cathepsin response to its advantage (27). A cathepsin Z haplotype was implicated as a genetic marker for tuberculosis susceptibility in a Ugandan household contact study (28); however, in a case-control study in Iran, the cathepsin Z rs34069356 polymorphism (29) was not associated with pulmonary tuberculosis. Finally, CG was found to be downregulated in \(M. tuberculosis\)-infected THP-1 cells, and this was associated with increased intracellular survival of \(M. tuberculosis\) (30).

In our own comprehensive survival study using mice deficient for CG, K, L, B, or Z, only mice deficient for serine protease CG succumbed to infection more rapidly than wild-type mice did, whereas mice deficient for the cysteine-type cathepsins did not show such an effect. This result indicates that there is a specific requirement for CG in long-term survival of mice that are aerogenically infected with \(M. tuberculosis\). CG/NE-double-deficient mice died at a similar rate as CG-single-deficient mice did. CG may therefore prove to be a useful marker for overall susceptibility to \(M. tuberculosis\) infection.

However, there was no discernible and consistent adverse effect on the bacterial load in infected organs of CG- or CG/NE–deficient mice compared with wild-type mice. Early in infection and shortly before death, bacterial loads in the lungs were identical in all three mouse strains examined. This is in contrast to BCG infection experiments performed with these mice, in which a slight

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** Bleeding time and plasma concentration of tissue factor pathway inhibitor in CG-KO, CG/NE-KO, and wild-type mice. (A) In vivo tail bleeding time in uninfected mice, during the chronic and late stage of infection; \(n = 15–19\) mice per group. (B) Analysis of plasma TFPI concentrations at day 139 postinfection. Data are representative of two independent experiments and represents mean ± SD of 15–19 (A) and 11–15 (B) mice per group. Statistical analysis was performed using Kruskal–Wallis and Dunn test. *\(p < 0.05\), **\(p < 0.01\).

![Figure 7](http://www.jimmunol.org/)

**FIGURE 7.** Treatment of \(M. tuberculosis\)-infected mice with liposomally encapsulated CG/NE. Wild-type mice were aerogenically infected with (A and B) 100 CFU or (C) 2000 CFU \(M. tuberculosis\) followed by treatment with CG/NE or PBS-loaded liposomes. (A) Intratracheal instillation of CG/NE-containing liposomes or PBS liposomes started at day 1 after low-dose infection and was repeated on days 7 and 14 postinfection. Pulmonary mycobacterial burden was determined on days 10 and 21 postinfection. (B) Repetitive orotracheal instillation of CG/NE containing or PBS liposomes at indicated days postinfection and analysis of pulmonary CFU counts on day 13 and 21 postinfection. (C) High-dose infection with \(M. tuberculosis\) followed by repetitive orotracheal instillation of CG/NE containing or PBS liposomes at indicated days postinfection and detection of pulmonary bacterial burden on day 14 postinfection. Data represent mean ± SD of 3–6 mice per group. Log-transformed data were statistically analyzed using two-way ANOVA and Bonferroni post hoc test.
(2-3-fold), yet significant growth enhancement in CG/NE–deficient mice was observed, concomitant with enhanced granulomatous inflammation and increased TNF levels in bronchoalveolar lavage fluids (10). Although M. bovis BCG is often used as a surrogate infection for tuberculosis, BCG is an attenuated vaccine strain that, because of differences in its cell wall make-up or intracellular localization, may be substantially more susceptible to direct enzymatic attack than M. tuberculosis.

There was a slight but significant reduction in the bacterial load in the spleens of infected moribund CG- and CG/NE–deficient mice compared with infected wild-type mice. In fact, spleens of CG- and CG/NE–deficient mice were enlarged. It is possible that the enhanced and dysregulated inflammatory response—as evidenced in this study by increased concentrations of TNF, IL-6, IL-1β, CXCL1, CCL2, and MIP-1α in the lungs and plasma of infected CG- and CG/NE–deficient mice—may have contributed to increased inflammatory cell influx also into the spleens, resulting in slightly enhanced bacteriocidal activity in this organ.

Efforts to uncover the cause of premature death of CG- and CG/NE–deficient mice revealed a modified chemokine and cytokine profile in the absence of these proteases during tuberculosis infection. Levels of proinflammatory cytokines and chemokines were significantly reduced in lung homogenates of CG- and CG/NE–KO mice early during infection, whereas the proinflammatory response was significantly increased in lung homogenates and plasma of these mice prior to death. This dysregulated profile was most notable for TNF, the key mediator in protection against and pathology of tuberculosis with pleiotropic functions. Perturbations of TNF levels severely affect the course of infection. Whereas low concentrations are associated with fatal disease, excessive TNF promotes immunopathology and induces a hyperinflammatory milieu (31). TNF exists as a proform bound to the surface membrane. Both CG and NE have been described to process membrane-bound TNF into a soluble and biologically active form in vitro (32). Conversely, CG and NE can degrade TNF, resulting in a loss of activity (32, 33). The overall in vivo effect of CG and NE is assumed to depend on the concentration of active enzymes in the microenvironment. Therefore, it is tempting to speculate that during the early phase of tuberculosis infection, the shedding activity of CG and NE dominates, resulting in a higher concentration of TNF in wild-type mice. During the late phase of murine tuberculosis, the degradation activity of both enzymes may prevail, dampening the TNF concentration in wild-type mice. Furthermore, CG and NE have been described to degrade and inactivate IL-6 at sites of inflammation (34). In addition, proteolytic processing of the chemokine MIP-1α by CG and NE abrogates its chemotactic activity (35). In line with these observations, the absence of CG or CG/NE resulted in significantly increased pulmonary IL-6 and MIP-1α levels during the late phase of tuberculosis infection. Consequently, our results indicate that neutrophil serine proteases may fine-tune the immune response during murine tuberculosis by proteolytic modification of proinflammatory cytokines and chemokines. Importantly, CG or CG/NE deficiency is associated with an unbalanced systemic hyperinflammatory response prior to death. Neutralization experiments using anti-chemokine or anti-chemokine Abs may be a useful tool to determine the role of individual factors or their combination in precipitating premature death of neutrophil serine protease deficient mice. Given the redundant and pleiotropic effects of the chemokines and cytokines putatively involved (i.e., TNF, IL-6 CCL2, MIP-1α, CXCL1), the administration of Abs must be carefully timed and orchestrated over a prolonged period; however, this is beyond the scope of this report.

Cytokines and chemokines help to control the formation and integrity of granulomas, the hallmark of tuberculosis infection (36). Although significant differences in chemokine and cytokine levels between wild-type and serine protease–deficient mice were observed, histopathologic examination revealed only minor differences in the pulmonary granulomatous response between CG- or CG/NE–deficient mice and the wild-type mice. After infection with M. bovis BCG, Steinwede et al. (10) observed a significantly increased granuloma formation in CG- and CG/NE–deficient mice but, even under these circumstances, no major differences were observed in the numbers and percentages of mononuclear phagocytes, neutrophils, and lymphocytes or in the mobilization of conventional lung dendritic cells toward the lung draining lymph nodes between wild-type and neutrophil serine protease deficient mice. We therefore did not perform flow cytometric analyses of lung tissue in our own experiments.

There was no measurable effect of the absence of CG or both, CG and NE, on TFPI concentration or M. tuberculosis dissemination in infected mice, although CG and neutrophil elastase have previously been demonstrated to regulate coagulation and intravascular thrombus formation and have been discussed as important for reducing dissemination of systemic bacterial infections (23). Interestingly, by investigating mice expressing either low or high levels of human TF instead of murine TF during experimental tuberculosis, it was recently shown that TF deficiency affects neither mycobacterial growth and dissemination nor the proinflammatory cytokine response to M. tuberculosis infection (37).

In summary, the ultimate cause of accelerated death in CG- and CG/NE–deficient mice infected with M. tuberculosis is likely unrelated to a direct antibacterial effect of CG/NE and potentially related to an unspecified dysregulated inflammatory response.

In vivo, intratracheal instillation or orotracheal administration of liposome-encapsulated CG and NE had no discernible effect on bacterial growth in the organs of M. tuberculosis–infected mice, even when treatment was prolonged for almost 3 wk with twice weekly applications starting as early as day 1 postinfection. This is in contrast to previous observations made with an identical protocol of liposome preparation and application in BCG-infected mice (10). It is possible that M. tuberculosis, in contrast to BCG, is not in a compartment accessible to liposomal CG/NE applied orotracheally. This might be because either a different cellular subset or a different intracellular compartment—compared with BCG—serves as the major habitat for M. tuberculosis during the early phase of infection (38). It is also possible that the phagolysosomal arrest induced by M. tuberculosis in the infected macrophage is so efficient that no colocalization of phagocytosed CG/NE–loaded liposome with M. tuberculosis in the same compartment occurs. On the other hand, the reduction of BCG bacterial load in vivo was reported to be in the order of 2–3fold, particularly during later times of infection (10). Because of the methodology for plating lung homogenates used in this study, this level of difference may have escaped detection or significance by statistical analysis.

Our data so far do not support the use of exogenous administration of CG and neutrophil elastase for immune-enhancing supportive treatment for M. tuberculosis infection.

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Disclosures

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