IL-18 and IL-12 synergy induces matrix degrading enzymes in the lung

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ABSTRACT

Interleukin (IL)-18 is a pro-inflammatory cytokine suggested to be involved in the development of pulmonary emphysema and inflammation. Studies involving immunology and cancer have revealed that IL-18 can have synergistic effects with IL-12. We have studied the presence of IL-18 and IL-12 receptors (IL-18R/IL-12R) in the lungs and whether IL-18 and IL-12, alone or in combination, have the ability to initiate the induction of mediators related to the development of emphysema and inflammation. The expression of the IL-18R was abundant in lungs compared to other organs (heart, liver, and spleen), and the IL-12R was also expressed in lung tissue. Mice treated with i.p. injection of recombinant murine IL-18 or IL-12 expressed significantly higher pulmonary mRNA levels of the matrix degrading enzymes metalloproteinase (MMP) 12 and cathepsin S, in addition to interferon-γ, tumor necrosis factor-α, and CXC chemokine ligand 9 (CXCL9) (all P < .05) than controls (received PBS). Treatment with IL-18 and IL-12 in combination showed an even more pronounced induction of these mediators, as well as a significant increase in MMP-9, IL-6, IL-1β, and transforming growth factor-β (P < .05). Furthermore, cellular apoptosis in lung tissue was induced. Immunohistochemical analysis revealed T-cell infiltration in pulmonary vessels following co-stimulation. In summary, IL-18 and IL-12 exert a synergistic effect on the lungs by inducing MMPs, cathepsins S, and pro-inflammatory cytokines, which may promote pulmonary emphysema and inflammation. The synergy between IL-18 and IL-12 involves infiltration of T-cells in the lungs, possibly induced by the T-cell chemoattractant CXCL9.

KEYWORDS cytokines, emphysema, inflammation, lung, synergy

INTRODUCTION

Interleukin (IL)-18 is a pro-inflammatory cytokine in the IL-1 superfamily [1–3], and it is structurally related to IL-1β. The activation of both cytokines is associated with the inflammasome NLRP3 [4]. The inflammasome contains caspase-1 that cleaves the biologically inactive precursor pro-IL-18 to biologically active IL-18, which is secreted from the cell. IL-18 was initially described as an inducer of interferon (IFN)-γ [5]. Subsequently, IL-18 has been shown to induce the expression of several other pro-inflammatory cytokines and chemokines, in addition to having the potential to attract T-cells [6–8]. In patients, elevated blood levels of IL-18 have been found in autoimmune diseases, acute graft-versus-host disease, hematological malignancies, sepsis, and inflammatory liver diseases, suggesting a fundamental role in immunity and inflammation [9–15]. With regard to lung diseases, a link between IL-18 and emphysema is supported by an experimental study in which transgenic mice overexpressing IL-18 developed pulmonary emphysema [16]. A possible role for IL-18 in human chronic obstructive pulmonary...
Cytokines, such as IL-1, contribute to the lung matrix causing pulmonary emphysema. and cathepsins are proteolytic enzymes that degrade various pro-inflammatory cytokines [26–31]. MMPs, such as MMP-9 and MMP-12, related to inflammation, and involves metallopro-teinases (MMPs) such as MMP-9 and MMP-12, which are known to be increased in COPD patients [19, 20] and alveolar hypoxia [25], which many COPD patients are repetitively exposed to.

COPD is a composite term comprising patients with emphysema and chronic bronchitis. The development of emphysema in COPD has causally been related to inflammation, and involves metalloproteinases (MMPs) such as MMP-9 and MMP-12, cathepsins, infiltration of inflammatory cells, and various pro-inflammatory cytokines [26–31]. MMPs and cathepsins are proteolytic enzymes that degrade the lung matrix causing pulmonary emphysema. Cytokines, such as IL-1β and TNF-α, are able to induce or activate these proteinases [32, 33]. The activity of MMPs is counteracted by tissue inhibitors of metalloproteinases (TIMPs) and the balance between MMPs and anti-MMPs is fundamental in the pathogenesis of emphysema [34].

Since there are indications of a link between IL-18, possibly in concert with IL-12, and the development of emphysema in both experimental and clinical studies, it would be of interest to study whether these cytokines in fact have the ability to induce the generation of mediators related to the emphysema development in the lungs. Thus, the aim of the present study was to investigate the early response of IL-18 with regard to induction of emphysema-related mediators, such as proteinases, and whether the up-regulation of mediators is potentiated by IL-12. In addition, we wanted to study the presence of IL-18 and IL-12 receptors in the lungs, compared to other organs, in order to obtain information about the lung as a possible target organ for these cytokines. To examine these aspects, the expression of the IL-18 receptor (IL-18R) and IL-12 receptor (IL-12R) in lung tissue from mice was determined. Subsequently, animals were injected with murine IL-18, IL-12, or IL-18 and IL-12 in combination, and induction of relevant MMPs, cathepsins, cytokines, and growth factors were examined by RT-PCR. The upregulated proteinases were also examined by Western analysis. In addition, histological and immunohistochemical (IHC) examination of lung tissue was performed.

### MATERIALS AND METHODS

#### Animals and Reagents

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Norwegian Animal Research Committee. Mice were obtained from Taconic, Ry, Denmark. The animals were housed with a day/night cycle of 12/12 hours at 21°C, and food and water were available ad libitum. A total of 60 7-week-old male C57Black/6j received injections with recombinant (r) murine (mu) IL-18 (0.5 µg b.i.d., B002-5, Medical & Biological Laboratories Co., Naka-ku Nagoya, Japan), IL-12 (1.0 µg, 419-ML-010, R&D Systems, Oxon, UK), or IL-18 (0.5 µg) and IL-12 (1.0 µg) intraperitoneally (i.p.) (n = 10 in all groups). The respective control groups (n = 10) received i.p. injections with phosphate-buffered saline (PBS). The doses of IL-18 (0.5 µg) and IL-12 (1.0 µg) were chosen according to previous studies [21, 35–37]. To the authors’ knowledge, serum levels following i.p. injection of IL-18 and IL-12 have not previously been published. In the present study, serum levels of IL-18 and IL-12 were measured with enzyme-linked immunosorbent assay (ELISA, R&D Systems, Oxon, UK), 24 hours after the animals received IL-18 + IL-12 (n = 6) or PBS (n = 6). Mice were euthanized by exsanguination drawing blood from inferior vena cava after rapid induction of general anaesthesia with isoflurane. The serum levels of both IL-18 (4328 ± 739 pg/mL in the IL-18 + IL-12 group versus 34 ± 8 pg/mL in the PBS group) and IL-12 (355 ± 26 pg/mL versus 249 ± 15 pg/mL) confirmed increased levels of the cytokines in the intervention group.

The mice were sacrificed for examination after 24 hours. To study the early response of IL-18 and IL-12 stimulation, 24 hours was chosen, after having observed a similar pattern of upregulation in the lungs in a pilot study with 24 hours and 7 days...
(data not shown). Six mice from each group were used for RT-PCR and Western analysis, while 4 mice from each group were used for histological and IHC examination. For RT-PCR and Western analysis, mice anesthetized with isoflurane were euthanized by dislocation of the neck, and the heart and lungs were rapidly excised and weighed. Total body weight was recorded before the procedure, and tibial length (TL) was measured. For histological and IHC examination, mice were euthanized by exsanguination drawing blood from inferior vena cava after rapid induction of general anesthesia with isoflurane. A midline sternotomy was performed and an endotracheal tube was inserted. The lungs were fixed and inflated by intratracheal instillation of 4% paraformaldehyde with a constant pressure of 25 cm H2O [38]. The extracted tissue was submerged in 4% paraformaldehyde. Eight mice did not receive any injections, and their organs were harvested for RT-PCR, Western blot analysis (n = 4), and IHC examination (n = 4) of the IL-18R and IL-12R.

Western Blot Analysis

Frozen tissues from liver, heart, spleen, and lung from healthy mice were homogenized on ice by using a Polytron 1200 homogeniser in buffer containing 210 mM sucrose, 2 mM EGTA, 40 mM NaCl, 30 mM HEPES, 5 mM EDTA, and Complete EDTA-free protease inhibitor cocktail tablet. Sodium dodecyl sulfate (SDS) was added to the homogenates to a final 1% concentration. Protein concentrations were quantified by using micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Homogenates were denatured at 100°C for 5 minutes in a sample buffer containing 50% sucrose, 7.5% SDS, 62.5 mM Tris-HCl, 2 mM EDTA, 0.2 M DTT, and 0.01% Bromphenol blue. Proteins were size-fractionated on 4%–15% SDS-page gels and blotted on to 0.45 µM PVDF-membranes, blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 for 1 hour at room temperature, then incubated with primary antibody overnight. A secondary antibody was added for 1 hour at room temperature. The primary antibodies used were anti-mouse IL-18Rα and anti-mouse IL12-Rβ1 (both from R&D systems, Oxon, UK). Recombinant IL-12Rβ1 (R&D systems, Oxon, UK) was used as standard for the IL-12R. Frozen lungs from each group treated with IL-18, IL-12, and IL-18 + IL-12 and their control group were prepared in the similar manner, and the primary antibodies used were anti-mouse MMP-12, anti-mouse MMP-9 (both from BioVision, CA, USA), and anti-mouse cathepsin-s (Abcam, Cambridge, UK), which were normalized to β-tubulin (Sigma-Aldrich, CA, USA). The secondary antibody used was anti-goat IgG HRP-linked whole antibody (R&D systems, Oxon, UK). The blots were developed by using ECLplus (GE Healthcare) and signals were quantified with ImageQuant software (GE Healthcare, Buckinghamshire, UK).

Real-Time Polymerase Chain Reaction

A RT-PCR system (ABI 7900HT Fast Real-Time PCR System, PE Biosystems, Foster City, CA, USA) was used to measure mRNAs. The specific mRNA transcripts were quantified by TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA, USA). Levels of IL-18Rβ (Mm00515172_m1) and IL-12Rβ1 (Mm00434189_m1) mRNA in the liver, heart, spleen, and lungs were determined. The mRNA amounts of MMP2 (Mm00439498_m1), MMP9 (Mm00600164_g1), MMP12 (Mm00500554_m1), neutrophil elastase (Mm00469310_m1), cathepsin B (Mm01310506_m1), cathepsin K (Mm00484039_m1), cathepsin S (Mm01255859_m1), TIMP-1 (Mm00441818_m1), TIMP-2 (Mm00441825_m1), TIMP-3 (Mm00441827_m1), TIMP-4 (Mm00446568_m1), Plasminogen activator inhibitor (PAI)-1 (Mm00435860_m1), interferon (INF)-γ (Mm00801778_m1), IL-1β (Mm01336189_m1), IL-6 (Mm00446191), tumor necrosis factor (TNF)-α (Mm00443258_m1), IL-13 (Mm00434204_m1), CXCL chemokine ligand 9 (CXCL9) (Mm00434946_m1), transforming growth factor (TGF)-β1 (Mm03024053_m1), type I collagen (Mm00483888_m1), and type III collagen (Mm00802331_m1) were measured in lung tissue. Total mRNA was isolated by using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). All RNA samples were quality assessed by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and RNA integrity numbers. The RNA samples were reverse transcribed by using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). All samples were tested in triplicate and average values were used for quantification. The average values were normalized to RPL32 mRNA (Mm02528467_g1) (Applied Biosystems, Foster City, CA, USA).

Histology

For histological analysis, 4 mice from every group were used. The lungs were sectioned transversely, stained with hematoxylin and eosin, and examined in a blinded manner by an experienced pathologist.
Immunohistochemistry

Formalin-fixed, paraffin-embedded sections from 4 mice in each group were prepared as previously described [39]. The sections were subjected to staining by using DAKO autostainer (DAKO, Glostrup, Denmark) and the following primary antibodies were used: polyclonal rabbit anti-mouse CD3 (1:50, Abcam, Cambridge, UK), monoclonal rat anti-mouse CD45R/B220 (1:4500), monoclonal rat anti-mouse forkhead-winged transcriptional factor box protein 3 (FoxP3; 1:50), monoclonal rat anti-mouse F4/80 (1:100; all from eBioscience, San Diego, CA, USA), polyclonal rabbit anti-mouse IL-18R (1:100, Santa Cruz Biotechnology, CA, USA), polyclonal goat anti-mouse IL-12R (1:100, Santa Cruz Biotechnology, CA, USA), polyclonal goat anti-mouse IL-12R (1:100, R&D Systems, Oxon, UK), and polyclonal anti-ACTIVE Caspase-3 (1:500, Promega, WI, USA). A total of 300 alveolar cells were counted in each lung section in a blinded fashion. To study apoptosis, the percentage of caspase-3 positive cells over total cells was determined, as previously described [40].

Statistical Analysis

Data are presented as means ± SE. Comparisons between groups were made by using unpaired student’s t-test or the nonparametric Mann-Whitney rank sum test depending on the distribution of data (SigmaStat 3.1.1, Systat Software, Richmond, CA, USA). Differences were considered significant for $P < .05$.

RESULTS

Animal Model

No significant changes in body weight were found in the IL-18, IL-12, or IL-18 + IL-12 stimulated groups compared to respective controls. All organ weights were related to TL. There were no differences between the stimulated groups and the controls with regard to the left ventricle, right ventricle, and lung weight normalized to TL.

IL-18 and IL-12 Receptors

High mRNA and protein levels of the IL-18R were measured in the lungs compared to liver, spleen, and heart (Figure 1A, C, and E). The most abundant level of IL-12R mRNA was observed in the spleen, followed by lung tissue, while the IL-12R protein level was highest in liver followed by spleen and lung (Figure 1B, D, and F). Immunohistochemical analysis showed expression of IL-18R and IL-12R in bronchial epithelium and in alveolar macrophages and lymphocytes. (Figure 2A, B, C and D). A higher number of mononuclear cells expressed IL-18R than IL-12R.

Matrix Metalloproteinases

Injection of IL-18, IL-12, and co-stimulation with IL-18 and IL-12 induced a significant increase in the gene expression of MMP-12 in lung tissue compared to controls (Figure 3A). On the protein level, both the inactive pro-form of MMP-12 (54 kDa) and the active forms of MMP-12 (45 kDa and 22 kDa) were higher in the co-stimulation group (Figure 3D and E). MMP-9 mRNA was also significantly increased after co-stimulation (Figure 3B), while the increase in the active form of MMP-9 protein (84 kDa) did not reach statistical significance (Figure 3F and G). No significant increase in mRNA levels of MMP-2 was found after treatment with IL-18 (130% ± 11% of control), IL-12 (123% ± 14% of control), or IL-18 + IL-12 (70% ± 15% of control). The amounts of neutrophil elastase mRNA were not changed after treatment with IL-18 (86% ± 23% of control), IL-12 (94% ± 27% of control), or IL-18 + IL-12 (87% ± 12% of control).

Cathepsins

Increased levels of cathepsin S mRNA in lung tissue were observed both after treatment with IL-18 and IL-12 separately, and in combination (Figure 3C). The inactive pro-form (37 kDa) and the active form of cathepsin S (24 kDa) are upregulated after co-stimulation with IL-18 and IL-12 (Figure 3H and I). No significant changes in the mRNA levels of cathepsin B were found after treatment with IL-18 (110% ± 8% of control), IL-12 (94% ± 14% of control), or IL-18 and IL-12 (125% ± 11% of control). Neither was the amount of cathepsin K mRNA significantly changed after injection of IL-18 (100% ± 6% of control), IL-12 (93% ± 12% of control), or IL-18 and IL-12 (114% ± 7% of control).

Antiproteases

Injection of IL-18 alone did not induce upregulation of antiproteases, while upregulation of TIMP-1 and TIMP-4 was observed following IL-12 stimulation (Figure 4A and D). Co-stimulation with IL-18 and IL-12 resulted in increased expression of TIMP-1, TIMP-3, and PAI-1 (Figure 4A, C, and E), whereas TIMP-2 was downregulated (Figure 4B).
Cytokines

IL-18 injection induced a significant increase in mRNA levels of INF-γ, TNF-α, and IL-10 in lung tissue compared to controls (Figure 5A, D, and E). Injection with IL-12 alone also increased the expression of INF-γ, TNF-α, and IL-10 mRNA compared to the control group (Figure 5A, D, and E). Stimulation with both IL-18 and IL-12 induced a pronounced increase in the pulmonary gene expression not only of INF-γ, TNF-α, and IL-10 but also of the inflammatory cytokines IL-1β and IL-6 (Figure 5A–E). The increase in IL-13 mRNA did not reach statistical significance after injection of IL-18 (153% ± 39% of control), IL-12 (148% ± 18% of control), or IL-18 + IL-12 (122% ± 12% of control).

Fibrosis Markers

We investigated the growth factor TGF-β and collagen type I and III. The gene expression of TGF-β was significantly increased after co-stimulation with IL-18 and IL-12 (Figure 5F). The increase of collagen I mRNA did not reach statistical significance after treatment with IL-18 (112% ± 9% of control), IL-12 (128% ± 16% of control), or IL-18 + IL-12 (103% ± 6% of control), neither did collagen III mRNA levels after stimulation with IL-18 (127% ±
12% of control), IL-12 (146% ± 25% of control), or IL-18 + IL-12 (106% ± 8% of control).

**CXCL9**

The gene expression of the T-cell chemoattractant CXCL9 was strongly induced in animals treated with IL-18 and IL-12 alone, compared to controls (Figure 6A). An even more pronounced increase was observed in animals co-stimulated with IL-18 and IL-12 (Figure 6A).

**Histological Analysis and Immunohistochemistry**

Treatment with IL-18 and IL-12 in combination induced infiltration of lymphocytes aggregating in the wall of pulmonary blood vessels. IHC examination revealed that the majority of the lymphocytes were T-cells, positively stained with CD3 (Figure 6B). Increased infiltration of B-cells, macrophages, or regulatory T-cells was not observed in any of the groups. Treatment with either IL-18 or IL-12 alone did not induce any histological changes compared to control animals.

**Apoptosis**

The percentage of caspase-3 positive cells, indicating apoptosis, was increased in the IL-18 + IL-12 group (25% ± 2% of total cell count) compared to controls (15% ± 1% of total cell count) ($P = .002$) (Figure 6D and E).

**DISCUSSION**

A high expression of IL-18 receptor in mouse lung tissue was observed, indicating the lung as a target organ for IL-18. The IL-12 receptor was also expressed in lung tissue, being a prerequisite for the synergistical effect of IL-12 and IL-18 in the lungs, as observed in the present study. Stimulation with IL-18 and IL-12 promoted an upregulation of the proteinases MMP9, MMP12, and cathepsin S that are all capable of degrading extracellular matrix in the lungs leading to
FIGURE 3. Pulmonary gene expression of MMP-12 (A), MMP-9 (B), and cathepsin S (C) in mice receiving injections with IL-18, IL-12, or IL-18 and IL-12 in combination (gray bars) compared with control animals receiving phosphate-buffered saline (PBS) (black bars). The mRNA levels in controls were set to 100%. Gene expression was analyzed by using quantitative RT-PCR. Pulmonary protein levels of pro-MMP-12, MMP-12 (45 kDa) and (22 kDa) (D, E), pro-MMP-9 and MMP-9 (84 kDa) (F, G), and procathepsin S and cathepsin S (24 kDa) (H, I) in co-stimulated group compared with control animals. Values are presented as mean ± SE. *P < .05, **P < .001 versus control mice.

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FIGURE 4. Pulmonary gene expression of TIMP-1 (A), TIMP-2 (B), TIMP-3 (C), TIMP-4 (D), and PAI-1 (E) in mice receiving injections with IL-18, IL-12, or IL-18 and IL-12 in combination (gray bars) compared with control animals receiving PBS (black bars). The mRNA levels in controls were set to 100%. Gene expression was analyzed by using quantitative RT-PCR. Values are presented as mean ± SE. *P < .05, **P < .001 versus control mice.

pulmonary emphysema [31, 41, 42]. IL-18 and IL-12 also induced an upregulation of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-γ that have been shown to promote pulmonary inflammation and to be of importance in the pathogenesis of emphysema [32, 33, 43–45]. Treatment with IL-18 and IL-12 increased the expression of the antiproteases TIMP-1, TIMP-3, and PAI-1, whereas the expression of TIMP-2 was decreased. Interestingly, signs of increased apoptosis in the lungs were observed as early as 24 hours following IL-18 and IL-12 injections, and increased apoptosis in the lungs is claimed to be of importance in the development of pulmonary emphysema [46]. Homing of T-cells to the lungs, possibly due to upregulation of the T-cell chemoattractant CXCL9, was a part of the pulmonary inflammation induced by IL-18 and IL-12.

In the present study, we found that IL-18 has the ability to induce gene expression of proteolytic enzymes known to participate in the pathogenesis of pulmonary emphysema. In addition, mRNA levels of several inflammatory cytokines, also linked to the emphysema development, were increased in the lungs following stimulation with IL-18. Co-stimulation with IL-18 and IL-12 demonstrated a synergistic effect on the induction both of proteolytic enzymes, their inhibitors, inflammatory cytokines, and the chemoattractant CXCL9. The synergy between IL-18 and IL-12 is known from interferon-γ induction in T-cells, where IL-12 upregulates the expression of the IL-18 receptor [5]. In addition, it has been shown that IL-18 and IL-12 production can stimulate synovial T-cells to produce more INF-γ in patients with rheumatoid arthritis, which in turn results in increased production of IL-1β and TNF-α leading to synoviocyte stimulation and joint destruction [47]. It has also been shown that prolonged co-stimulation with IL-18 and IL-12 is lethal in mice, and that the lungs of these mice show infiltration of mononuclear leukocytes [21]. Generation of IL-18 in COPD has been located to bronchial epithelial cells, T-cells, and macrophages [17]. Macrophages have also been shown to produce IL-12 [48]. The mechanism for the increased levels of these cytokines has not been fully elucidated, but with regard to IL-18, it has been shown that cigarette smoke, known to promote emphysema, may activate the innate immune system leading to the conversion of pro-IL-18 to its

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biologically active form [20]. Macrophages, which express both IL-18 and IL-12, also have the ability to produce several of the other mediators described in the present study, that is, IFN-γ, TNF-α and MMP12, MMP9, and cathepsin S [17, 48, 49]. Interestingly, these cells also express the receptors of IL-18 and IL-12, as shown in the current study. If the synergy between IL-18 and IL-12 participates in the pathogenesis of emphysema, macrophages may play a central role by both being target cells of these cytokines and a source of emphysema promoting proteases and cytokines.

It has, to the authors' knowledge, not previously been shown that IL-18 and IL-12 in synergy might induce pulmonary generation of proteases and inflammatory mediators strongly linked to the development of emphysema. MMP-9 is related to the development of emphysema in humans through increased plasma levels of MMP-9 observed both in α1-antitrypsin deficiency-associated emphysema and in emphysema related to smoking [50, 51]. Also, smoking leads to increased MMP-9 protein levels and activity in induced sputum [52]. Hence, increased levels of both IL-18 and IL-12 [19, 53] in lungs exposed to cigarette smoke may induce pulmonary MMP-9 production that could participate in the development of emphysema. Smokers with severe COPD have increased MMP-9 mRNA in pulmonary tissue, and MMP-9 correlates negatively with FEV1, diffusion capacity of carbon monoxide, and the partial pressure of oxygen, indicating that MMP-9 is related to the severity of COPD [54]. The link between MMP-9 and emphysema was strengthened by a study showing that mice overexpressing human MMP-9 develop emphysema [42]. To our knowledge, it has not previously been shown that IL-18 and IL-12 synergy induces increased expression of MMP-9 in the lungs, suggesting a link between these cytokines and MMP-9, which might be of importance in the process of extracellular matrix degradation in the lungs.

MMP-12 has a broad and potent matrix degrading capacity, which can be induced by cigarette smoke [55]. In experimental studies, MMP-12 seems to play a central role in the development of emphysema [56, 57]. In mice, knockout of MMP-12 protects against smoke-induced emphysema [41], and guinea pigs treated with a MMP-9/MMP-12 inhibitor did not develop emphysema after exposure to cigarette smoke [26]. With regard to human emphysema, however, data on MMP-12 are not that easy to
interpret [58]. Some studies report increased levels of MMP-12 in sputum and alveolar macrophages from smokers and COPD patients [59, 60], while other studies have not been able to confirm these findings [61, 62]. In the present study, we found that stimulation with IL-18 and IL-12, both separately and in combination, resulted in increased MMP-12 expression, which, at least in mice, may contribute to the development of emphysema.

In addition to MMPs, the cystein proteinases cathepsins have the potential of degrading extracellular matrix, leading to pulmonary emphysema [63, 64]. Cathepsin S in particular, seems to play a role in the development of emphysema by promoting matrix degradation and cell apoptosis [31, 65]. In our study, cathepsin S was induced both by IL-18 and IL-12, and in combination the increase was pronounced, underlining the synergy between IL-18 and IL-12. It is possible that cathepsin S is a link between these cytokines and the observed signs of apoptosis in the present study [31]. Taken together, we have shown that IL-18 and IL-12 stimulation initiates production of the important matrix degrading enzymes MMP-9, MMP-12, and cathepsin S in the lungs, all having the potential to induce pulmonary emphysema.

The observed up- and downregulation of TIMPs and PAI-1 may influence the proteolytic effect on the lungs. Recent studies, however, show that prolonged stimulation with IL-18 in mice overexpressing this cytokine in the lungs leads to the development of severe emphysema, indicating a shift in the balance between proteases and antiproteases in the direction of increased proteolytic activity [19, 66]. The importance of IL-18 in the development of emphysema has recently been highlighted, but the mechanism by which IL-18 leads to emphysema is not completely understood [66, 67]. By showing generation of the matrix degrading enzymes MMP-9, MMP-12, and cathepsin S, our result may possibly contribute to the understanding of mechanisms participating in this complex process. The role of IL-12 in the development of emphysema is not clear, but in addition to having a synergistic effect with IL-18 on the generation of matrix degrading enzymes and emphysema promoting cytokines, as shown in the present
investigation, increased levels of IL-12 in COPD has been documented [23, 68]. In experimental models, it has been shown that IL-18 and IL-12 given both intraperitoneally and locally in the airways induce inflammatory changes in the lungs, and both elevated circulating and pulmonary levels of IL-18 and IL-12 have been observed in COPD patients [17, 21, 23, 69–72]. Thus, both systemic and local increase in the levels of IL-18 and IL-12 may promote pulmonary inflammation.

The inflammation occurring in pulmonary emphysema involves invasion of inflammatory cells and production of pro-inflammatory cytokines in the lungs. We found that IL-18 and IL-12 induced infiltration of T-cells in the lungs, possibly via the T-cell chemoattractant CXCL9, which was substantially increased by stimulation with these cytokines. CXCL9 can be involved in homing of T-cells to the lungs in COPD [73]. Interestingly, patients with pulmonary emphysema have increased numbers of T-cells that produce the pro-inflammatory cytokine IFN-γ [30], which was markedly increased by the synergy between IL-18 and IL-12 in the present study. IFN-γ may promote pulmonary emphysema by inducing cathepsin S-dependent apoptosis, and also through MMP-12 in an apoptosis-independent pathway [31, 44]. In our study, both pathways could be induced by IL-18 and IL-12. The powerful pro-inflammatory cytokines IL-6, IL-1β, and TNF-α, which were all induced by IL-18 and IL-12 synergy, have also been linked to the development of inflammation and emphysema in the lungs [32, 33, 43, 45]. IL-6, IL-1β, and TNF-α have the potential to upregulate MMPs, and for instance, when IL-1β production is ectopically expressed in the lung epithelium, it causes increased expression of MMP-9 and MMP-12 and lung pathology that resembles emphysema [32]. MMP-9 is capable of activating IL-1β from its biologically inactive form, and instillation of MMP-12 into mouse airways induced an acute inflammatory response with leukocyte invasion, accompanied by increased pro-inflammatory cytokines such as IL-6 and TNF-α, and also MMP-9 [74]. The pro-inflammatory cytokine IL-13 has previously been linked to the development of pulmonary inflammation and emphysema in transgenic mice overexpressing IL-18 [16]. The increase in IL-13 following stimulation with IL-18 and IL-12 in the present study, however, did not reach statistical significance. According to previous studies, the interaction between pro-inflammatory cytokines, cathepsins, and MMPs suggests a complex network of mediators participating in the development of pulmonary emphysema, which in humans takes years to develop [16, 31–33, 36–40]. The results of the present study suggest that IL-18 and IL-12 may take part in this process, but it should be noted that the duration of the study was too short to discover possible histological signs of emphysema. The 24 hours model only elucidates the acute responses induced by these two cytokines, and further studies using established mouse models of COPD and examinations of human tissues and cells are necessary to clarify the relevance of IL-18 and IL-12-induced pathways in the development of emphysema.

IL-18 and IL-12 stimulation induced the anti-inflammatory cytokine IL-10, which has the ability to suppress pro-inflammatory cytokines such as TNF-α, IL-6, and IFN-γ [75, 76]. IL-10 may also decrease the expression of MMPs [28, 77]. In the present study, the anti-inflammatory effects of IL-10 were not able to inhibit a robust induction of pro-inflammatory cytokines, MMPs, and cathepsin S. Patients with COPD have reduced concentrations of IL-10 in sputum, which might be a mechanism for increasing lung inflammation [78]. Thus, the process of emphysema might both be initiated by an inflammatory response that causes destruction of pulmonary tissue or by reducing the defenses, such as a reduction in IL-10, which could protect lung tissue during inflammation [28, 77]. An additional component in the development of emphysema is claimed to be an altered repair mechanism [79, 80]. The growth factor TGF-β, which was modestly increased by the synergy between IL-18 and IL-12, is claimed to be involved in repair of lung tissue through stimulation of collagen production [81]. In our study, however, collagen type I and III was not upregulated by IL-18 and IL-12. Although fibroblasts from patients suffering from COPD can produce increased amounts of TGF-β, they seem to be less responsive to effects of TGF-β, thereby exhibiting a diminished repair response, which could contribute to the development of pulmonary emphysema [79].

In summary, IL-18 and IL-12, which have been found in increased levels in patients with COPD, may promote the development of pulmonary emphysema by induction of matrix degrading enzymes MMP9, MMP12, and cathepsin S and by inducing cellular apoptosis. The inflammatory response observed, involved influx of T-cells, possibly mediated through the chemokine CXCL9, and induction of pro-inflammatory cytokines in mouse lungs. These effects seem to be mediated through the IL-18 receptor, which is abundantly expressed in lungs compared to other organs, and the IL-12 receptor, which is also present in lung tissue.

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