The Journal of Immunology

IL-8 Dictates Glycosaminoglycan Binding and Stability of IL-18 in Cystic Fibrosis

Emer P. Reeves, Michael Williamson, Barry Byrne, David A. Bergin, Stephen G. J. Smith, Peter Greally, Richard O'Kennedy, Shane J. O'Neill and Noel G. McElvaney

J Immunol 2010; 184:1642-1652; Prepublished online 21 December 2009;
doi: 10.4049/jimmunol.0902605
http://www.jimmunol.org/content/184/3/1642

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

IL-8 Dictates Glycosaminoglycan Binding and Stability of IL-18 in Cystic Fibrosis

Emer P. Reeves,*[,1] Michael Williamson,*[,†,1] Barry Byrne,[‡] David A. Bergin,* Stephen G. J. Smith,[§] Peter Greally,[†] Richard O'Kennedy,[‡] Shane J. O'Neill,* and Noel G. McElvaney*

Dysregulation of airway inflammation contributes to lung disease in cystic fibrosis (CF). Inflammation is mediated by inflammatory cytokines, including IL-8, which illustrates an increase in biological half-life and proinflammatory activity when bound to glycosaminoglycans (GAGs). The aim of this project was to compare IL-8 and IL-18 for their relative stability, activity, and interaction with GAGs, including chondroitin sulfate, hyaluronic acid, and heparan sulfate, present in high quantities in the lungs of patients with CF. Bronchoalveolar lavage fluid was collected from patients with CF (n = 28), non-CF controls (n = 14), and patients with chronic obstructive pulmonary disease (n = 12). Increased levels of IL-8 and reduced concentrations of IL-18 were detected in bronchial samples obtained from CF individuals. The low level of IL-18 was not a defect in IL-18 production, as the pro- and mature forms of the molecule were expressed and produced by CF epithelial cells and monocytes. There was, however, a marked competition between IL-8 and IL-18 for binding to GAGs. A pronounced loss of IL-18 binding capacity occurred in the presence of IL-8, which displaced IL-18 from these anionic-matrices, rendering the cytokine susceptible to proteolytic degradation by neutrophil elastase. As a biological consequence of IL-18 degradation, reduced levels of IL-2 were secreted by Jurkat T lymphocytes. In conclusion, a novel mechanism has been identified highlighting the potential of IL-8 to determine the fate of other inflammatory molecules, such as IL-18, within the inflammatory milieu of the CF lung. ***The Journal of Immunology*****, 2010, 184: 1642–1652.**

The pathogenesis of lung disease in cystic fibrosis (CF) has not been fully characterized; however, proinflammatory cytokine dysregulation and neutrophil-dominated inflammation play major roles. Dysregulation of cytokine production, with a comparative defect in Th1 immune response, has been identified as a cause of pulmonary inflammation (1, 2). In support of this theory, disproportionate concentrations of proinflammatory cytokines have been recorded in CF bronchial samples, including elevated levels of the potent neutrophil chemoattractant IL-8/CXCL8 (3, 4) and, in contrast, dramatically diminished levels of the IFN-γ–inducing factor IL-18 (5, 6).

IL-8 is markedly increased in CF bronchial samples, where it contributes to the transendothelial migration of neutrophils into the CF airways (7). This massive infiltration of neutrophils leads to the generation of reactive oxygen species (8) and release of proteolytic enzymes, including neutrophil elastase (NE), which is central to the lung injury observed in patients with CF (9). In vitro studies have illustrated that glycosaminoglycan (GAG) molecules, including heparan sulfate (HS) moieties of proteoglycans, act as consensus binding sites for IL-8 (10, 11). Binding to GAGs renders IL-8 impervious to proteolysis, thus increasing the half-life and activity of the chemokine at the site of inflammation, consequently contributing to sustained neutrophil recruitment in CF (12, 13).

In contrast to IL-8, IL-18 levels are greatly reduced in CF bronchial samples. IL-18 contributes to a number of inflammatory disorders, including rheumatoid arthritis (14), Crohn's disease, and sarcoidosis (15). Overproduction of IL-18 is associated with the pathogenesis of chronic obstructive pulmonary disease (COPD) (16). Mice deficient in IL-18 have impaired IFN-γ production, and macrophages of these animals fail to promote Th1 responses in vitro (17). Furthermore, IL-18 binding protein (IL-18BP) isoforms IL-18BPa and IL-18BPb have been shown to neutralize the biological activity of IL-18 (18).

IL-18 is expressed as a 24-kDa inactive precursor (pro–IL-18) molecule, which is cleaved by caspase-1 to present the active 18-kDa mature protein. Caspase-3 and neutrophil proteases generate inactive peptides from pro–IL-18 (19), suggesting that at sites of inflammation, IL-18 activity may be regulated by neutrophil-derived proteins. Despite this reasoning, a rationale as to why reduced levels of IL-18 persist in lungs of patients with CF has not been fully elucidated. One investigation has shown that stimulation of CF PBMCs with IL-10 decreased IL-18 expression, thus proposing that IL-10 may inhibit IL-18 in CF mononuclear cells (6). As an alternative

*Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital; [†]Department of Respiratory Medicine, Adelaide and Meath Hospital, Incorporating the National Children's Hospital, Tallaght; [‡]Applied Biochemistry Group, School of Biotechnology, Dublin City University; and [§]Department of Clinical Microbiology, St. James's Hospital, Trinity College Dublin, Dublin, Ireland

[1]E.P.R. and M.W. contributed equally to this work.

Received for publication August 7, 2009. Accepted for publication November 20, 2009.

This work was supported by the Medical Research Charities Group/Health Research Board, the Children's Research Centre, Crumlin Hospital, the Alpha-1 Foundation, and the Program for Research in Third Level Institutes administered by the Higher Education Authority.

Address correspondence and reprint requests to Dr. Emer P. Reeves, Respiratory Research Division, Department of Medicine, Royal College of Surgeons in Ireland, Education and Research Centre, Beaumont Hospital, Dublin 9, Ireland. E-mail address: emerreeves@rcsi.ie

Abbreviations used in this paper: A1AT, α-1 antitrypsin; ACT, α-1 antichymotrypsin; APRO, aprotinin; BALF, bronchoalveolar lavage fluid; Benz, benzamidine; CF, cystic fibrosis; CG, cathepsin G; CMK, N-(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone; COPD, chronic obstructive pulmonary disease; CS, chondroitin sulfate; FEV_1, forced expiratory volume in 1 s; GAG, glycosaminoglycan; HA, hyaluronic acid; HS, heparan sulfate; IL-18BP, IL-18 binding protein; NE, neutrophil elastase; OP, orthophenanthroline; PBST, PBS containing 0.01% (v/v) Tween 20; PE, *Pseudomonas* elastase; PR3, proteinase 3; Rh, recombinant human; RU, response unit; SBTI, soybean trypsin inhibitor; SLPI, secretory leukocyte protease inhibitor; TLCK, Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK, N-p-Tosyl-L-phenylalanine chloromethyl ketone.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902605

explanation it has also been speculated that *Pseudomonas aeruginosa*-secreted exotoxin A may also decrease IL-18 expression (20).

The present study was undertaken to compare the presence of IL-18 to IL-8 and to fully determine molecular factors impacting upon low IL-18, yet contrastingly elevated IL-8 levels within the inflammatory milieu of the CF lung.

Materials and Methods

Chemicals

All chemicals and reagents were the highest purity and were purchased from Sigma-Aldrich, Dublin, Ireland, unless indicated otherwise. Experiments employed carrier-free recombinant human (Rh)IL-8 and RhIL-18 (MBL Medical & Biological Laboratories, Nagoya, Japan).

Bronchoalveolar lavage fluid sample collection

Bronchoalveolar lavage fluid (BALF) samples were collected from patients in the Adelaide and Meath National Children's Hospital. Ethical approval was obtained from the St. James's Hospital/Adelaide and Meath National Children's Hospital Research Ethics Committee, and full informed parental consent was obtained prior to bronchoscopy. All procedures were carried out by the respiratory pediatric consultants of the National Children's Hospital, as previously described (21). In the CF group ($n = 28$), 11 were chronically colonized by *P. aeruginosa* [average age 14.47 ± 1.41 y, average forced expiratory volume in 1 s (FEV_1) 64.65 ± 6.84% (mean ± SE)], and 17 were noncolonized (average age 7.78 ± 2.11 y, average FEV_1 61.00 ± 8.637%). Patients with CF were deemed noncolonized when no *P. aeruginosa* growth was detectable in sputum or cough swabs during the previous 12 mo. In the non-CF control group, the average age was 8.21 ± 1.22 y ($n = 14$) and FEV_1 was 96 ± 6.59%. For the COPD group ($n = 12$), mean age was 51.5 ± 2.65 y and FEV_1 was 52.25 ± 8.27% predicted. BALF was centrifuged at 2000 × *g* for 10 min and the supernatant aliquoted and stored at −80°C. The BALF cell pellet was resuspended in physiological saline, and cell counts were performed using trypan blue exclusion. Differential cell counts were performed manually after staining with May-Grunwald and Giemsa with at least 200 cells counted per slide.

Cytokine quantification by ELISA

Concentrations of IL-18, IL-18BPa, IL-8, and IL-2 in BALF, serum, and cell culture samples were measured by ELISA (R&D Systems, Minneapolis, MN), conducted in accordance with the manufacturer's instructions. Cytokine-specific Abs were employed: mouse IgG_1 antihuman IL-18, mouse IgG_1 antihuman IL-18BPa, mouse IgG_1 antihuman IL-8, and mouse IgG_{2A} antihuman IL-2 (R&D Systems). IFN-γ concentration was measured using a Human IFN-γ Immunoassay Kit (R&D Systems). BALF samples were measured for protein with the Bio-Rad protein assay (Bio-Rad, Hertfordshire, U.K.) and cytokine quantities among patient samples expressed as picograms per milligram of BALF protein.

SDS-PAGE and Western blot analysis

Proteins were separated by electrophoresis in 12% NuPAGE gels (Invitrogen, Carlsbad, CA). For Western blot analysis, proteins were transferred to polyvinylidene difluoride membrane at 100 mA for 1 h using a semidry electrophoretic blotting system (Sigma-Aldrich). Specific goat polyclonal Abs were used to detect IL-18 and GADPH (Santa Cruz Biotechnology, Heidelberg, Germany). Mouse mAbs to pro–IL-18 and IL-8 (R&D Systems) were employed using the manufacturer's recommended concentrations. After three 10-min washes in PBS containing 0.01% (v/v) Tween 20 (PBST), the nitrocellulose was incubated with anti-goat IgG HRP-linked Ab (Santa Cruz Biotechnology) or anti-mouse IgG HRP-linked Ab (Cell Signaling Technology, Danvers, MA). Immunoreactive protein bands were visualized employing SuperSignal West Pico chemiluminescent substrate (Pierce Chemical Co, Rockford, IL) after exposure to Kodak X-Omat LS film (Sigma-Aldrich). In some experiments, immunoreactive bands were quantified by densitometry using the Syngene G:BOX Chemi XL gel documentation system (Syngene, Cambridge, U.K.).

Production of pro–IL-18 and secretion of mature IL-18 by purified CF and control healthy monocytes

Monocytes were isolated from heparinized venous peripheral blood obtained from patients with CF or healthy controls. Under sterile conditions, cells were purified by dextran sedimentation and Lymphoprep centrifugation (Axis-Shield, Cambridgeshire, U.K.). The mononuclear cell interphase was removed and washed with HBSS (Life Technologies). Subsequently, monocytes were purified to 97% purity using the EasySep human CD14 selection mixture (StemCell Technologies, London, U.K.). Monocytes were suspended in 1 ml PBS containing 2% (v/v) FCS and 1 mM EDTA.

For the detection of intracellular pro–IL-18, monocytes (5×10^5) were resuspended in radioimmunoprecipitation assay buffer (250 μl), sonicated 2 × 5 s, and centrifuged (200 × *g* for 5 min at 4°C). Supernatants (20 μl) were subjected to SDS-PAGE and Western blot analysis, as already described.

For measurement of IL-18 secretion by monocytes, cell culture conditions and protocol for cell activation were as previously described (22). In brief, monocytes (5×10^5) seeded into 24-well plates were incubated for 24 h within media [HBSS plus 2% (v/v) FCS] alone or media containing *P. aeruginosa* LPS (1 μg/ml). Cells were centrifuged (500 × *g*, 5 min) and the supernatant removed for the detection of IL-18 by ELISA.

RT-PCR analysis for pro–IL-18 and caspase-1 gene expression

The CFBE41o- (CFBE) cell line was generated by transformation of CF bronchial cells with SV40 and is homozygous for the ΔF508 mutation. The 16HBE14o- (HBE) is a non-CF human bronchial epithelial cell line. These were obtained as a gift from D. Gruenert (University of Vermont, Burlington, VT) (23) and were cultured as previously described (24). The CF C38 cell line was transfected with wild-type adeno-associated viral CFTR and stably expresses wild-type CFTR (25). The latter was kindly supplied by P. Zeitlin at Johns Hopkins School of Medicine, Baltimore, MD. Total RNA was extracted using TRI Reagent according to the manufacturer's instructions. Prior to cDNA synthesis, RNA was treated with DNase I. cDNA synthesis from mRNA (1 μg) was performed using the SuperScript kit (Invitrogen, Paisley, U.K.) using oligo(dT) primers. PCR was performed using AccuTaq polymerase TaqDNA polymerase, 1× PCR buffer, and 10 mM dNTPs (Promega, Southampton, U.K.) in a 50 μl volume containing 100 pmol each of the following primers: IL-18: 5′-CAGCCGCTTTAGCAGCCA-3′, 5′-CAAAGGAATTGTCTCCCAGTGC-3′; β-actin: 5′-GGGTACATGGTGGTGCCG-3′, 5′-GCCGGGAAATCGTGCGTG-3′; and caspase 1: 5′-GTTTCTTGGAGACATCCC-3′, 5′-TAATGTCCTGGGAAGAGG-3′. PCR conditions were as follows: 95°C denaturing for 5 min (95°C denaturing for 30 s, 55°C annealing for 30 s, 72°C extension for 6 min) × 28 cycles; and 72°C extension for 6 min. PCR products were resolved on a 1% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide.

Jurkat T cells and IL-2 production

For the measurement of IL-2 production by human lymphoblastic Jurkat E6.1 T cells, cell culturing conditions and concentrations of cell activators were as previously described (15). Cells (5×10^5 per well) were exposed to RhIL-12 (10 ng/ml; R&D Systems) for 24 h and then for a further 24 h with 30 ng/ml RhIL-18 or 30 ng/ml RhIL-18 predigested with NE (10^{-6} M; Elastin Products Company, Owensville, MO) for 30 min. Culture supernatants were removed and analyzed for the presence of IL-2 by ELISA. Controls for this experiment included untreated cells, PMA (50 ng/ml) activated cells, and cells exposed to the NE inhibitor pefabloc (20 mM).

Proteolytic degradation of IL-18

RhIL-18 (64.5 or 400 pg) was incubated with 20 μl of CF or healthy control BALF, and levels of exogenously added IL-18 measured by ELISA after 1 h incubation at 37°C. Time course experiments were performed by incubating RhIL-18 with control or CF BALF for 0, 10, 15, 20, 25, 30, and 60 min or 1, 2, 8, and 24 h at 37°C and proteolytic digestion of IL-18 analyzed by Western blot analysis. In some cases, CF BALF was incubated at 37°C for 1 h with the following protease inhibitors before addition of RhIL-18: 13 μM aprotinin (APRO), 5 mM benzamidine (Benz), 0.15 mM Nα-Tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 0.5 mM N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 20 mM N-(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (CMK), 10 μM soybean trypsin inhibitor (SBTI), 20 mM orthophenanthroline (OP), 1 μM recombinant human elafin (Proteo Biotech AG, Kiel, Germany), 1 μM α-1 antichymotrypsin (ACT), 1 μM α-1 antitrypsin (A1AT), 10 mM EDTA, 20 μM GM6001 (Merck Biosciences, Nottingham, U.K.), and 4 μM secretory leukocyte protease inhibitor (SLPI) (R&D Systems). RhIL-18 (100 ng) was then added and the solution incubated at 37°C for 1 h. Reactions were stopped by the addition of SDS-PAGE sample buffer and boiled for 3 min. The samples were then tested for the presence of IL-18 by gel electrophoresis and Western blot analysis as described above.

For further analyses, 1×10^{-5} to 1×10^{-12} M NE (Athens Research and Technology, Athens, GA), proteinase 3 (PR3) (Athens Research and Technology), cathepsin G (CG) (Elastin Products Company), or *Pseudomonas* elastase (PE) (metalloproteinase also known as pseudolysin; Elastin Products Company) was added to 100 ng IL-18 in PBS. Reactions were

incubated at 37˚C and samples collected at specific time points and subjected to Western blot analyses. In some reactions, the effect of GAGs on NE-mediated IL-18 digested was tested. As HS (13), chondroitin sulfate (CS) (26), and hyaluronic acid (HA) (27) are all increased in patients with CF, a mixture of all 3 GAGs was employed. IL-18 (100 ng) was preincubated for 30 min with HS, CS, and HA (from human umbilical cord) at a ratio of 1:10 (w/w) for each GAG prior to proteases exposure. The ratio of protein to GAGs employed was based on concentrations previously described (21).

NE activity assay

NE activity in BALF samples from *Pseudomonas*-colonized and uncolonized patients was estimated employing the substrate N-(Methoxysuccinyl)-Ala-Ala-Pro-Val *p*-nitroanilide as previously described (21). For some reactions, the effect of GAGs on NE activity was tested by preincubating NE for 1 h with HS, CS, and HA at a ratio of 1:10 or 1:100 (w/w) for each GAG.

Biacore-based analysis of interactions

All surface-plasmon resonance assays were performed on a Biacore 3000 instrument using a CM5 carboxymethylated dextran sensor chip (GE Healthcare, Buckinghamshire, U.K.). Protocols used were as previously described (28). All analyses were performed in triplicate on 3 consecutive d (interday), and binding responses (response unit [RU]) were monitored as a function of GAG concentration.

Coating of polystyrene beads with GAGs and flow cytometric binding assay

Coating of polystyrene beads was as follows. Polystyrene beads 10 μm in diameter (Polyscience, Eppelheim, Germany) (200 μl) were pelleted by centrifugation, resuspended in 500 μl of a 1 mg/ml solution of HS, CS, and HA in Buffer A (15 mM Na_2CO_3 and 35 mM $NaHCO_3$ [pH 9.3]), followed by end-over-end rotation overnight at 20˚C. Beads were washed in Buffer A, then washed in PBST, and resuspended and stored in 200 μl of PBS containing 5% (v/v) glycerol at 4˚C.

Beads coated with GAGs (20 μl) were washed in 20 mM sodium citrate buffer (pH 6.3) and incubated with RhIL-18 (100 ng, 500 ng, or 1 μg) at ambient temperature for 30 min. Additionally, the ability of IL-8 to compete and displace IL-18 from GAGs was determined by exposing the GAG-coated polystyrene beads to RhIL-18 (500 ng) simultaneous with IL-8 (250 ng or 500 ng) or by adding IL-8 1 h after IL-18. Reactions were stopped by the addition of 2% (v/v) formaldehyde, and unbound protein was removed by washing three times in PBST. Adsorption of IL-18 to the beads was determined by staining the beads with a goat polyclonal IgG Ab to IL-18 (Santa Cruz Biotechnology) and an anti-goat IgG (Santa Cruz Biotechnology) conjugated to FITC in PBST containing 1% (w/v) BSA. The specificity of the interaction between IL-18 and GAGs was tested by incubating GAG-coated polystyrene beads with anti–IL-18 and FITC-labeled secondary Ab alone. Reactivity of the beads with the Ab was assessed by flow cytometry, and a total of 10,000 gated events were collected. Analysis of the data was performed with the CellQuest software program from BD Biosciences.

Displacement of IL-18 from GAGs

Prior to each assay, wells from Nunc 96-well microtiter plates were coated with 1 mg/ml of HS, CS, and HA in Buffer A at 20˚C overnight. Wells were washed three times with PBST. The presence of GAGs on the microtiter plate was confirmed by a chromatic method with toluidine blue staining. Human recombinant proteins were prepared in 20 mM sodium citrate buffer (pH 6.3), and plates were exposed to RhIL-18 (25 ng or 6 ng) simultaneous with IL-8 (25 ng) or IL-8, RANTES, or MCP-1α (25 ng of each) (R&D Systems) added 1 h post IL-18 addition. In a subset of experiments, IL-18 (25 ng) was pretreated with 2 mM pyridoxal 5′-phosphate or 2 mM 2,3-butanedione as previously described (29, 30). The plates were incubated on a platform-rotating incubator for 1 h at room temperature. Unbound recombinant protein was removed by washing three times in PBST. Adsorption of IL-18 to the GAG-coated plate was determined by probing the wells with a goat polyclonal IgG Ab to IL-18 (Santa Cruz Biotechnology) followed by biotinylated anti-goat IgG in PBST and 1% (w/v) BSA. The plates were washed three times with PBST, and 100 μl of HRP-conjugated streptavidin was added to each well for 15 min and peroxidase activity was measured by the addition of ABTS. The color was allowed to develop for 5 min, and the absorbance was measured at 450 nm. The controls for this displacement ELISA assay were the GAG-coated wells containing all reagents except primary and/or biotinylated secondary Ab and the GAG-coated wells incubated only with HRP conjugate.

Statistical analyses

The data were analyzed with the GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA) and results expressed as means ± SEM. The Mann-Whitney *U* test or Kolmogorov-Smirnov two sample tests were employed to identify significant differences. A p value < 0.05 was deemed significantly different.

Results

Comparative quantification of IL-8 and IL-18 in CF serum and BALF samples

Quantification of cytokines by ELISA revealed elevated levels of IL-8 in CF and COPD BALF compared with control subjects (Fig. 1*A*). In contrast, CF BALF IL-18 levels were consistently low and equal to the control group (Fig. 1*B*). COPD and CF IL-8 serum levels were elevated above control values (Fig. 1*C*). In addition, CF and COPD IL-18 serum levels were higher than the control group but also statistically different from each other (Fig. 1*D*).

Despite low levels of IL-18 in CF BALF, high levels of the inhibitory protein IL-18BP isoform IL-18BPa (18) were detected in CF and COPD BALF compared with controls. In addition, low levels of IFN-γ were observed in control and CF BALF compared with COPD samples (Fig. 1*F*).

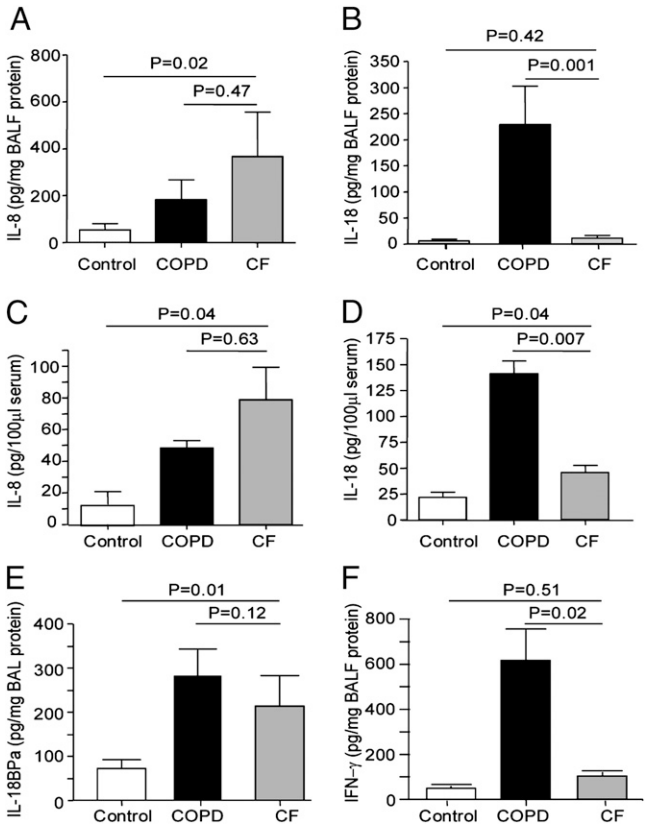

FIGURE 1. Protein levels of inflammatory molecules in BALF and serum from CF, COPD, and healthy controls. *A* and *B*, Protein levels of IL-8 and IL-18 in BALF of control subjects ($n = 12$) and CF ($n = 15$) and COPD ($n = 12$) individuals. *C* and *D*, Protein levels of IL-8 and IL-18 in serum of control ($n = 5$) and CF ($n = 8$) and COPD ($n = 5$) individuals. *E* and *F*, IL-18BPa and IFN-γ levels in BALF of control ($n = 5$) and COPD ($n = 12$) and CF ($n = 8$) subjects ($n = 12$). Protein levels are shown as picogram per milligram of BALF and levels in serum shown as picogram per 100 μl serum. Data are mean ± SE for three determinations and two-tailed p value calculated by unpaired t test. Statistical p values illustrated on each graph.

Pro–IL-18 gene expression, protein levels, and processing to IL-18 is equal in control and CF airway cells and circulating monocytes

To evaluate why CF individuals had low levels of IL-18 protein in CF BALF, RT-PCR analysis was performed. An amplicon corresponding to IL-18 confirmed the presence and expression of IL-18 in LPS-stimulated (1 μg/ml) HBE, CFBE and C38 cell lines (Fig. 2*A*). For comparative expression analysis, simultaneous expression of the pro–IL-18 processing enzyme, caspase-1, was performed with equal expression detected in all three human airway cell lines tested.

Next, we determined relative protein expression levels of pro–IL-18 in control and CF blood monocytes ($n = 3$) by conventional Western blot analysis. Immunodetection of pro–IL-18 showed equal levels of monocyte intracellular pro–IL-18 protein (Fig. 2*B*) when normalized in relation to the GADPH loading control ($p = 0.31$).

The possibility that reduced levels of IL-18 within CF lung samples was a result of defective processing of pro–IL-18 was explored and excluded on the basis that addition of LPS to the medium of cultured control and CF monocytes increased the release of mature IL-18 in the surrounding media of both cell types (Fig. 2*C*). Taken together, these results confirm that CF cells express IL-18 on both the gene and protein level.

Degradation and clearance of mature IL-18 in CF BALF by serine protease

The effect of CF BALF on IL-18 was assessed to determine its ability to cleave or degrade the cytokine. ELISA was employed to detect RhIL-18 added to control and CF BALF. As shown in Fig. 3*A*, almost all RhIL-18 added to control BALF was recovered. However, under the same experimental conditions, the lower concentration of RhIL-18 (64 pg) added to CF BALF was undetectable, and only 16.4% of the higher concentration (400 pg) was recovered after a 1-h incubation at 37°C. A time course experiment was performed by incubating RhIL-18 with control or CF BALF for 1, 2, 8, or 24 h at 37°C. As shown in Fig. 3*B*, levels of RhIL-18 detected in CF BALF by Western blot analysis decreased over time. A further time course of 10, 15, 20, 25, 30, or 60 min revealed that IL-18 is rapidly proteolytically digested within 60 min.

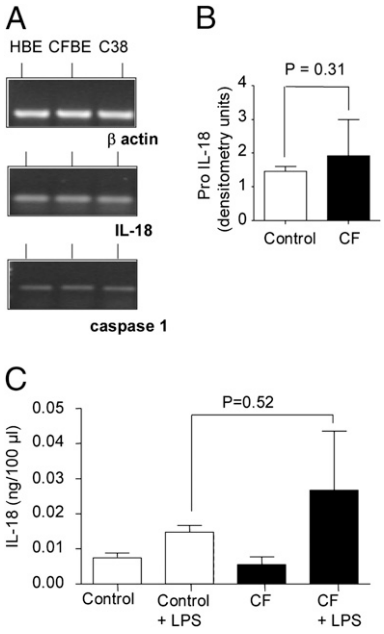

FIGURE 2. IL-18 gene and protein expression. *A*, RT-PCR analysis of the housekeeping gene β-actin, pro–IL-18, and caspase-3 following exposure of HBE, CFBE, and C38 cells to LPS (1 μg/ml) for 24 h. In two other experiments, similar expression profiles were obtained and revealed equal levels of pro–IL-18 and caspase-1 gene expression within the three cell types. *B*, Electrophoretically separated control and CF monocytic cell lysate was transferred to nitrocellulose and probed with rabbit antiserum to pro–IL-18. Western blot densitometry values were normalized against corresponding GAPDH immunoreactive bands and revealed similar levels of monocyte intracellular pro–IL-18 protein ($p = 0.31$). *C*, The effect of LPS (1 μg/ml) on secretion of mature IL-18 by control and CF cultured monocytes. Each measurement is the mean ± SE of triplicate experiments and the non-statistically different p value of 0.52 calculated by two-tailed t test.

To identify protease(s) responsible for cleavage and ultimate destruction of IL-18 in CF BALF, various protease inhibitors were preincubated with CF BALF before adding RhIL-18. The use of broad range protease inhibitors targeting elastase-like, trypsin-like, chymotrypsin-like proteases, and metalloproteases identified elastase-like proteases as playing a key role in the proteolytic cleavage of IL-18. As shown in Fig. 3*C*, CMK, elafin, A1AT, and SLPI inhibited the cleavage of IL-18, whereas Benz, SBTI, TLCK (trypsin-like), APRO, ACT, TPCK (chymotrypsin-like), and GM6001 and OP (metalloproteinase-like) had no effect. Taken together, these results show that elastase-like proteases, in particular NE, PR3, and CG, may be involved in the degradation of IL-18 within the CF lung.

Identification of proteinase in CF BALF responsible for destruction of IL-18

To challenge the concept that NE, CG, and/or PR3 cleave mature IL-18, dose-response incubations were performed by incubating RhIL-18 with 10^{-12}–10^{-5} M of NE, CG, or PR3 for 2 h at 37°C. As shown in Fig. 4*A*, human NE and CG cleaved IL-18 at an enzyme:protein substrate molar ratio of 3.6:1, whereas in contrast, PR3 appeared less effective in cleaving IL-18. As illustrated in Fig. 4*B*, NE (10^{-6} M) cleaved RhIL-18 quickly with cleavage products of molecular mass 12 kDa and 9 kDa detected after just 2.5 min incubation. In contrast, over the same time course (0–20 min), CG had no proteolytic effect on IL-18.

Given that elastase is the main neutrophil serine protease responsible for the proteolytic cleavage of IL-18 in CF BALF, the level of NE in CF BALF was estimated and correlated with the low level of IL-18 (Fig. 4*C*). Once the level of NE increased >~0.15 μM within the CF BALF, there was no observable IL-18. Next, we measured NE levels in both *P. aeruginosa*-colonized and noncolonized CF BALF, and, as shown in Fig. 4*D*, the concentration of cell-free elastase increased from 0.19 ± 0.06 μM to 0.69 ± 0.2 μM BALF protein in *P. aeruginosa*-colonized compared with noncolonized CF BALF. Additionally, cell-free elastase activity corresponded to decreased levels of IL-18 (Fig. 4*D*), with an approximate 53.06% reduction in detectable levels of the cytokine in *P. aeruginosa*-colonized CF BALF.

As *P. aeruginosa*-colonized CF BALF had lower levels of IL-18, we also investigated the ability of PE to cleave IL-18 in vitro. RhIL-18 was incubated with 10^{-12}–10^{-5} M purified protease and analyzed by Western blot with anti–IL-18 Ab and, as shown in Fig. 4*E*, PE was found to completely cleave RhIL-18 at an enzyme:protein substrate molar ratio of 0.36:1. Time course incubations with PE in excess (10^{-6} M) were preformed over 80 min at 37°C, and, under these conditions, almost complete cleavage of IL-18 was observed after 80 min of incubation. Collectively, these results confirm the degradation of IL-18 by both bacterial and neutrophil-derived proteases.

With regard to IL-18 degradation and molecular mechanisms of IL-18 signaling, we investigated the impact of IL-18 degradation on its biological activity. Production of IL-2 by Jurkat T cells (15) was employed to determine the significance of NE digestion on IL-18 activity (Fig. 4*F*). IL-2 secretion was measured in cell culture

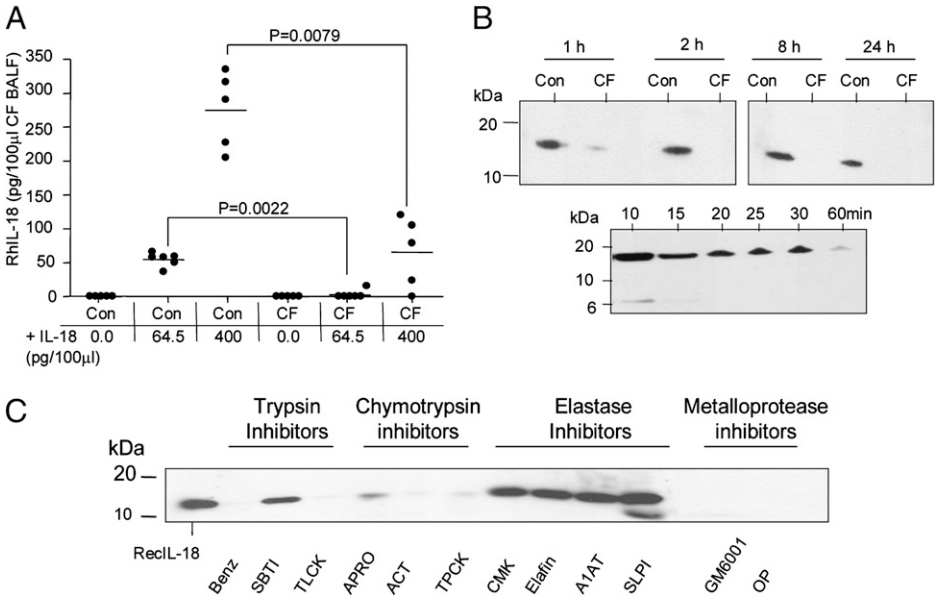

FIGURE 3. IL-18 is proteolytically degraded in CF BALF. *A*, RhIL-18 (64.5 or 400 pg) was added to 100 µl of control (Con) or CF BALF. Reactions were incubated for 1 h at 37°C and IL-18 level detected employing a sandwich-based ELISA. Low levels of IL-18 were recovered in CF BALF. *B*, RhIL-18 (10 ng) was added to control (Con) or CF BALF (20 µl) and incubated at 37°C for 1, 2, 8, or 24 h (*top panel*) or 10, 15, 20, 25, 30, and 60 min (*bottom panel*). Samples were electrophoresed on a 12% SDS-PAGE, followed by Western blot analysis employing mouse polyclonal Ab raised against IL-18. *C*, CF BALF (20 µl) was preincubated for 1 h at 37°C with protease inhibitors, 5 mM Benz, 10 µM SBTI, 0.15 mM TLCK, 13 µM APRO, 1 µM ACT, 0.5 mM TPCK, 20 mM CMK, 1 µM elafin, 1 µM A1AT, 4 µM SLPI, 20 µM GM6001, or 20 mM OP, followed by the addition of IL-18 (10 ng) for 1 h. Each blot is representative of one out of three experiments.

supernatants after activation of T cells with phorbol ester (PMA) or IL-12 followed by IL-18. After PMA or IL-18 and IL-12 activation, large amounts of IL-2 were secreted by T cells. Elastase degradation of IL-18 led to a loss of IL-2–inducing ability, as there was no significant difference between T cells exposed to IL-12 or IL-12 and IL-18 predigested with NE in levels of IL-2 production. Within this experiment, IL-12 was added 24 h prior to NE treated IL-18 and therefore unexposed to NE; however, to ensure that NE degradation of IL-18 led to decreased levels of IL-2 and not direct degradation by NE, the serine protease inhibitor pefabloc (2 µM) was included and had no effect on spontaneous IL-2 release (data not shown).

Proteolytic degradation of IL-18 is delayed by binding to GAGs

Binding of IL-8 to GAGs within lung tissue has previously been reported (31), and for this reason, a Biacore-based assay was developed to compare and contrast the interaction of IL-8 and IL-18 with GAGs. The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride/n-hydroxysuccinimide–based coupling and ethanolamine capping led to the capture of 3402 RU of IL-8 corresponding to ~3.4 ng/mm^2 and 4.2 ng/mm^2 of IL-18 (4180 RU). As HS (13), CS (26), and HA (27) are increased in patients with CF, comparative interday analysis of the interaction of a mixture of all three GAGs with both IL-18 and IL-8 was performed. Fig. 5*A* shows that GAGs bound to both IL-8 and IL-18 in a concentration-dependent manner with a greater degree of binding to IL-8 than IL-18 (mean RU values 52.9 ± 3.13 and 15.1 ± 3.10, respectively, for 200 µg/ml GAGs). In addition, documented pH measurements of CF airways range from 5.88 (32) to 6.61 (33), and, for this reason, binding of IL-8 and IL-18 to GAGs was examined at the median value of pH 6.3. The effect of pH 6.3 on protein-GAG binding is illustrated in Fig. 5*A*, and under these conditions, a significant increase in binding signal was observed. Comparative statistical analysis was performed for monitoring the interaction between IL-8 and the GAG mixture at pH 7.4 and 6.3, with an approximate 150% (100 µg/ml GAGs) and 115% (200 µg/ml GAGs) increase in binding recorded at pH 6.3 ($p = 0.002$ and 0.003, respectively). The reduction in pH to 6.3 also had a positive effect on IL-18 binding, as results revealed an 86% (100 µg/ml GAGs) and 102% (200 µg/ml GAGs) increase in binding at pH 6.3 ($p = 0.09$ and 0.01, respectively). The binding of IL-18 to GAGs is a novel observation, and, therefore, a second method employing FACS analysis was performed to further illustrate this specific interaction. Fig. 5*B* indicates that an increase in the interaction of IL-18 on GAG-coated polystyrene beads at pH 6.3 was observed in a dose-dependent manner (100 ng, 500 ng, and 1 µg). Nonspecific absorption on beads exposed to Ab alone was negligible.

To investigate the possibility that positively charged amino acid residues located on the surface of IL-18 (34) play important roles in electrostatic binding to GAGs, experiments used chemical modification of arginine and lysine residues by 2,3-butanedione and pyridoxal 5′-phosphate respectively. As illustrated in Fig. 5*C*, extensive inhibition of binding (~60%) was obtained with 20 mM pyridoxal 5′-phosphate, whereas 2,3-butanedione gave only 40% inhibition. In addition, treatment of IL-18 by a combination of both 2,3-butanedione and pyridoxal 5′-phosphate significantly reduced the interaction of IL-18 ($p = 0.02$), thus suggesting that both arginine and lysine residues are in part responsible for regulating IL-18:GAG interactions.

HS and heparin were previously shown to enhance the activity of IL-8 (35), and hence the effect of GAG binding on the activity of IL-18 was investigated. Experiments were designed to determine the ability of IL-18 to induce IL-2 production by Jurkat T cells. IL-2 secretion was measured in cell culture supernatants after exposure of T cells to IL-12 for 16 h, followed by IL-18 on its own or IL-18 prebound to GAGs. Following activation, no significant difference was observed in the level of IL-2 produced by T cells exposed to IL-18 or GAG-bound IL-18 employed at either a 10:1 or 100:1 GAG: cytokine ratio (190 ± 0.15 pg/ml, 200 ± 46 pg/ml, $p = 0.94$; 125 ± 50 pg/ml, $p = 0.23$, respectively; results not shown).

It has previously been reported that binding to GAGs induces structural stabilization of IL-8 in the inflamed lung (10). For this reason, the ability of NE to degrade IL-18 in the presence and

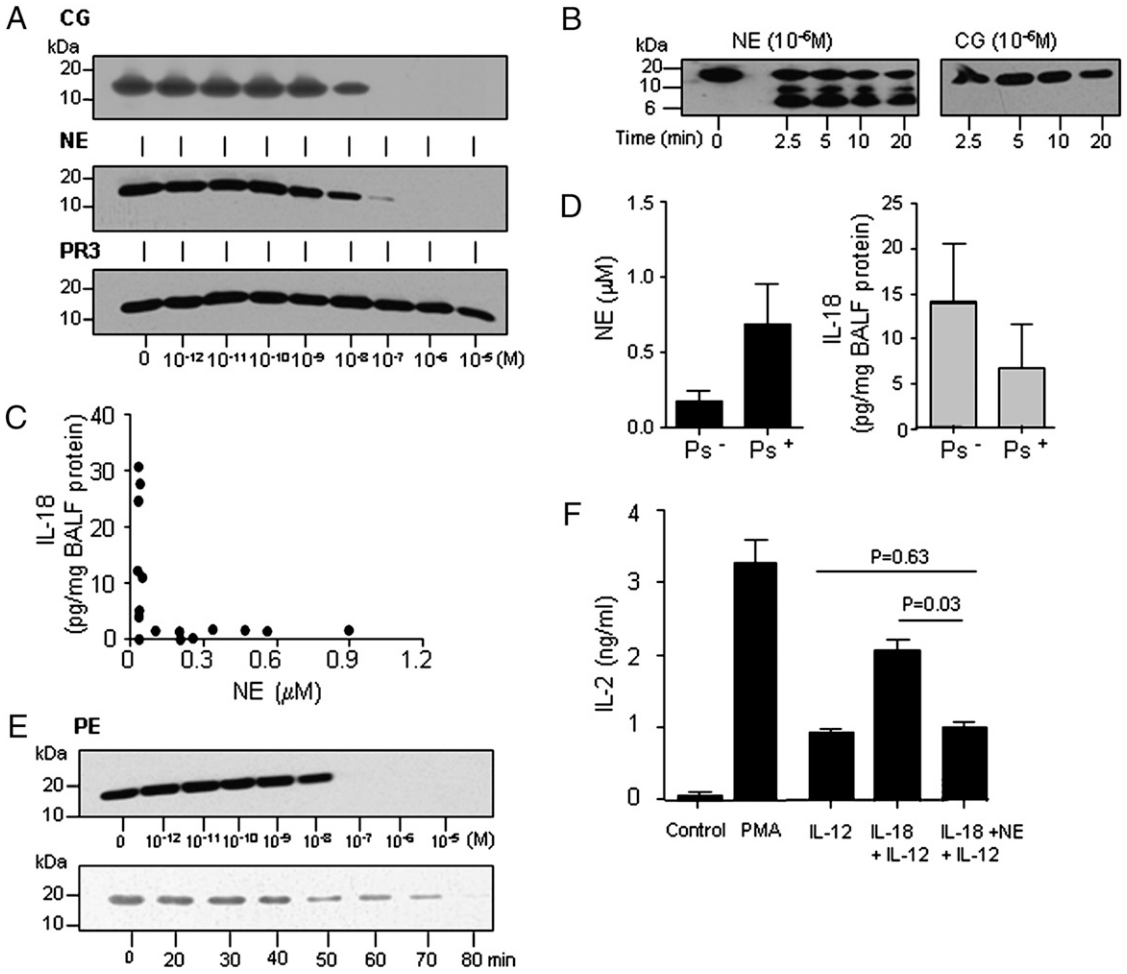

FIGURE 4. Neutrophil serine protease and PE degrade RhIL-18. *A*, Dose-response incubations of RhIL-18 (100 ng) with CG, NE, or PR3 (10^{-12}–10^{-5} M) for 2 h. *B*, Time course (0–20 min) incubations of NE and CG (10^{-6} M) with IL-18 (100 ng) at 37°C. Samples were electrophoresed and analyzed by Western blot using polyclonal Ab to IL-18. NE most effectively degraded IL-18. *C*, Threshold effect of NE activity leading to reduced levels of IL-18 in CF BALF. *D*, NE activity and IL-18 levels in *Pseudomonas* colonized (Ps^+) CF BALF samples ($n = 17$) compared with noncolonized CF (Ps^-) ($n = 9$). Low levels of IL-18 correlated with increased NE activity in *Pseudomonas*-colonized CF samples. *E*, Dose-response (10^{-12}–10^{-6} M) and time course (0–80 min) incubations of the degradation of RhIL-18 (100 ng) by PE. Each blot is representative of one out of three experiments. *F*, The biological relevance of IL-18 degradation. IL-12 (10 ng/ml) pretreated Jurkat T cells were cultured with a final concentration of 30 ng/ml RhIL-18 either untreated or pretreated with 10^{-6} M NE for 30 min at 37°C. NE IL-18 processing caused diminished IL-2 production ($p = 0.03$). Results shown are means ± SE of triplicate experiments.

absence of GAGs was investigated. A time course experiment was performed by incubating RhIL-18 (100 ng) with NE (10^{-6} M) in the presence and absence of GAGs employed at a 10:1 ratio of GAGs:IL-18, for 5, 10, or 20 min at 37°C. Fig. 5*D* shows that levels of RhIL-18 detected by Western blot analysis decreased over time, but the rate of proteolysis was greatly reduced by the inclusion of GAGs. The effect of GAGs in vitro in this proteolytic system was found to be significant with a 62.26 ± 4.42% decrease in the level of IL-18 degradation observed at the 20 min time point ($p < 0.05$) (Fig. 5*E*). In addition, activity of NE was not inhibited at the concentration of GAGs employed within these experiments (result not shown). Taken together, these results indicate that GAG-immobilized IL-18 maintains activity, and the described interaction protects this cytokine against proteolytic degradation. Accordingly, these results do not constitute a rationale for a lack of IL-18 within the CF lung.

IL-8 competitively competes and displaces IL-18 from GAGs

Because IL-8 interacts with proteoglycans with high affinity, we hypothesized that IL-8 may interfere with the presentation of IL-18 on GAGs, rendering the cytokine susceptible to NE-mediated proteolysis. To challenge this hypothesis, NE digestion of IL-18 was carried out in the presence and absence of GAGs and also in the presence of IL-8. A time course experiment was performed by incubating RhIL-18 (100 ng) with GAGs (10:1 ratio) and NE (10^{-6} M) in the presence of RhIL-8 (100 ng) for 5, 10, or 20 min at 37°C. The rapid proteolytic digestion of IL-18 was prevented in the presence of GAGs, but this effect was reversed by inclusion of RhIL-8, and proteolysis of IL-18 in the presence of IL-8 was consistently observed (Fig. 6*A*). To exclude the possibility that fluctuation in reaction rate occurred as a result of imbalance between substrate molecule and enzyme caused by NE targeting of IL-8, a Western blot of reactions probed for RhIL-8 revealed that degradation of IL-8 did not occur over the time course employed (Fig. 6*A*, *lower panel*).

In order to quantify competitive binding of IL-8 and IL-18 to GAGs, an ELISA-based assay was designed employing GAG-coated microplates and a polyclonal anti–IL-18-specific Ab. Surfaces coated with GAGs at a concentration of 30 μg/ml were capable of binding ~62 ± 6.8 pg IL-18/cm^2 (Fig. 6*B*). The binding capacity of GAG-coated surfaces did not decrease after repeated washing with detergent solutions [e.g., PBS containing 0.05% (v/v) Tween 20 (result not shown)]. Hence, GAGs did not appear to leach from the surface during assays.

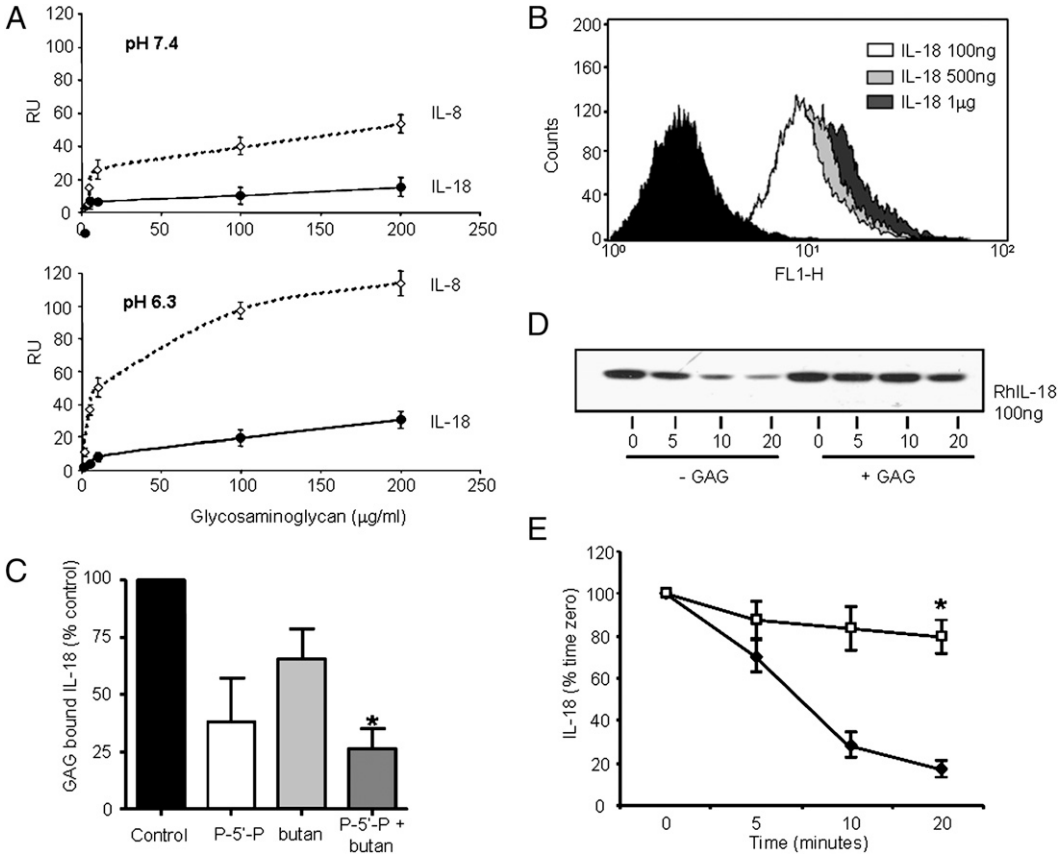

FIGURE 5. IL-18 is resistant to proteolytic degradation when bound to GAGs. *A*, Comparative binding of GAGs (mixed HS, CS, and HA) to IL-8 and IL-18 at pH 7.4 and pH 6.3. Increasing concentrations of GAGs (1, 5, 10, 100, and 200 μg/ml) were injected over an IL-8 (hatched line) or IL-18–immobilized sensor chip surface. Values of the vertical axis, expressed in RUs, represent increases in mass concentration on the IL-8 and IL-18–immobilized sensor surfaces because of pH-sensitive binding of GAG molecules. *B*, Flow cytometry analysis of binding of IL-18 to polystyrene GAG-coated beads (10 μm) was assessed at pH 6.3. Abs specific to IL-18 were used to identify beads that were positive for bound protein. The isotype control Ab is illustrated in black (filled). Representative results from one of three separate experiments is shown. *C*, GAGs (1 mg/ml of HS, CS, HA) were immobilized on a 96-well microtiter plate and the binding of RhIL-18 (25 ng) detected (Control, expressed as 100%). Modification of lysine residues by 2 mM pyridoxal-5′-phosphate (P-5′-P) or arginine residues by 2 mM butanedione (butan) reduced the level of detectable IL-18. Each measurement is the mean ± SE of triplicate experiments. $*p = 0.02$ compared with control. *D* and *E*, RhIL-18 (100 ng) pre-exposed to GAGs at a ratio of 1:10 (w/w) was protected from NE (10^{-6} M)-mediated proteolysis. Each reaction was performed in triplicate and subjected to SDS-PAGE and densitometry of immunoblots revealed a significant reduction in the level of IL-18 degradation at the 20-min time point. $*p < 0.05$.

The exposure of GAG-coated surfaces to IL-8 (25 ng), together with IL-18 (25 ng), competitively reduced the level of detectable IL-18 by ~57% with 26.25 ± 2.4 pg IL-18/cm^2 detected ($p < 0.05$). This competitive effect was more evident when IL-8 (25 ng) was employed in excess concentration over IL-18 (6 ng). Under these conditions, IL-8 competitively reduced the level of detectable IL-18 by ~82.52% (Fig. 6*B*).

These in vitro assays also demonstrated that IL-18 could be competitively displaced from its GAG binding site by IL-8. Within this reaction, IL-8 (25 ng) was added to the microplate 1 h after the addition of IL-18 (25 ng), and under these displacement conditions, the level of IL-18 detected was reduced by ~32% with 42 ± 3.7 pg IL-18/cm^2 detected. In addition, IL-18 was abolished to undetectable levels when excess IL-8 (25 ng) was added 1 h after the addition of IL-18 (6 ng) (Fig. 6*B*). Controls for this experiment included primary and/or secondary Ab with negligible levels of nonspecific interaction to GAG matrices detected.

A second approach was undertaken to demonstrate the ability of IL-8 to compete and displace IL-18 from GAG surfaces (Fig. 6*C*). The bead-binding capacity of polystyrene beads coated with GAGs revealed that detection of IL-18 (500 ng) was not affected by addition of IL-8 (250 ng) added either simultaneously or 1 h later. However, an increasing concentration of IL-8 (500 ng) added at the same time as IL-18 (500 ng) competed for binding to the immobilized GAGs, significantly reducing the level of bound IL-18. Summation of the curves employing Kilmogorov-Smirnov statistics revealed a D/s(n) (an index of similarity) value of 28.45 ($p < 0.001$ between levels of bound IL-18 in the presence and absence of IL-8). Results also revealed that IL-8 (500 ng) added 1 h after the addition of IL-18 significantly displaced and reduced the level of detectable IL-18 [D/s(n) = 11.32; $p = 0.001$]. Polystyrene beads of the described reactions were subsequently subjected to Western blot-based analysis (Fig. 6*D*), with a detectable increase in the level of IL-8 bound to GAG-coated beads when added simultaneously or 1 h post IL-18 addition. In further support of our finding, a rank of GAG-binding affinity has been previously proposed whereby RANTES > IL-8 > MIP-1α (12), and, as illustrated in Fig. 6*E*, IL-18 was equally displaced from GAGs by IL-8 and MIP-1α, with a significantly greater proportion of IL-18 displaced by RANTES ($p < 0.006$).

We interpret this data to suggest that competition by inflammatory mediators, in particular IL-8, found in high levels in the CF lung, results in the diminished binding of IL-18 to GAGs and indicates that the described competition may play a role in the deficiency of IL-18 within the CF lung.

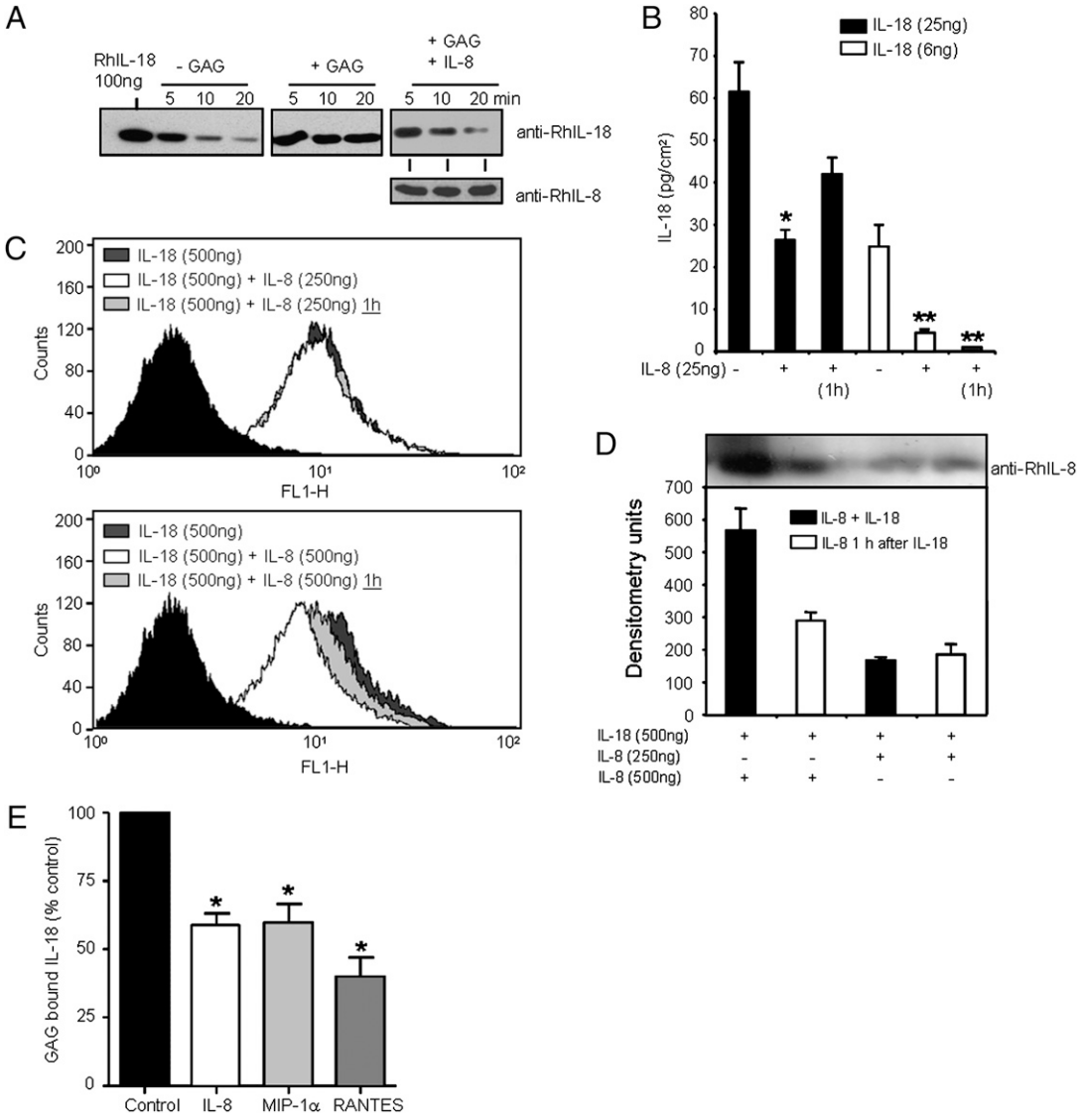

FIGURE 6. IL-8 competitively binds and displaces IL-18 from GAG binding sites. *A*, A time course experiment was performed by preincubating RhIL-18 (100 ng) with NE (10^{-6} M) in the presence or absence of GAGs (10:1 ratio) or RhIL-8 (100 ng) for 5, 10, or 20 min at 37°C. Proteolysis of IL-18 and stability of IL-8 (*bottom panel*) was determined by Western blot analysis. NE (10^{-6} M) digestion of IL-18 was prevented in the presence of GAGs, an effect overturned in the presence of IL-8. *B*, GAGs (1 mg/ml of HS, CS, and HA) were immobilized on 96-well microtiter plates and the binding of RhIL-18 (25 ng or 6 ng) to immobilized GAGs detected in the presence or absence of IL-8 (25 ng) added together with IL-18 or 1 h later. IL-8 competitively displaced IL-18 from GAGs. Three independent sets of binding reactions were performed, one of which is presented. $*p < 0.05$ compared with 25 ng IL-18; $**p < 0.05$ compared with 6 ng IL-18. *C*, Binding of IL-18 (500 ng) to polystyrene GAG-coated beads was assessed in the presence of IL-8 (250 ng or 500 ng). IL-8 (500 ng) added simultaneously or 1 h after the addition of IL-18 significantly reduced the level of detectable IL-18 ($p < 0.001$). Representative results from one of three separate experiments are shown. *D*, Polystyrene GAG-coated beads of reactions described in *C* were analyzed by Western blot and revealed positive binding of IL-8. *E*, GAGs (1 mg/ml of HS, CS, and HA) were immobilized on a 96-well microtiter plate and the binding of RhIL-18 (25 ng) detected (Control). IL-8, MIP-1α, or RANTES (25 ng) added 1 h after IL-18 significantly reduced the level of detectable IL-18 ($*p = 0.02$; $*p = 0.05$; and $*p = 0.006$, respectively). Bar graph measurements are the mean ± SE of triplicate experiments. *p* values were calculated by two-tailed *t* test.

Discussion

Reduced IL-18 protein levels have been described in CF BALF samples (5, 6), and, by way of explanation, inhibition of IL-18 expression by IL-10 was proposed (6). Unexpectedly, however, expression profiles within CF lung epithelial cells and macrophages revealed abundant IL-18 compared with lungs from control subjects (5). The apparent contradiction between reduced levels of IL-18 in CF BALF and abundant IL-18 lung cell expression was addressed in this study. We demonstrate that the pro- and mature forms of IL-18 are expressed and produced equally by CF and control epithelial cells and monocytes. However, once secreted into the extracellular milieu of the CF lung, the cytokine is rapidly degraded by neutrophil serine and bacterial proteases. GAGs are capable of binding IL-18, and, while bound, the cytokine is impervious to proteolytic degradation. The stability of IL-18 is, however, provisional and undermined in the presence of IL-8, which competes and displaces IL-18 from its GAG binding site. Reduced resistance and Th1 response to microbial infection have been correlated with IL-18 deficiency (36), and hence this study suggests that a strategy leading to a reduction in levels of IL-8 may positively affect IL-18 stability and improve the pulmonary outcome of *P. aeruginosa*-infected CF individuals. However, the impact of increased IL-18 on the progression of CF lung disease is not clear-cut, especially as we have detected inhibitory IL-18BPa within the CF airways. Consequently, restored IL-18 levels may only affect the immune response at a time when concentrations achieved overwhelm IL-18BPa inhibitory activity.

Inflammation is recognized as a major pathogenic element of CF lung disease. In fact, a growing body of data has emerged supporting the belief that inflammatory dyregulation in CF may occur early in life preceding infection and may be prompted by noninfectious stimuli (37). In addition, a fundamental intrinsic abnormality of inflammatory cells, including epithelial cells (1), neutrophils (38), lymphocytes, and granulocytes (39), has been proposed as a leading cause of inflammation. Our results confirm previous observations that CF cells express IL-18 at both the gene and protein level (5). Elevated IL-18 levels were detected in CF serum samples but, in total contrast, bronchoalveolar lavage findings in patients with CF suggested either minimal expression or incessant depletion of IL-18 from the CF lung. In addition, IL-18BPa (18), which regulates the contribution of IL-18 to Th1 responses in vivo, was detected in CF BALF, raising the question as to why an inhibitory protein is expressed in the absence of its cognate substrate. Our results demonstrate that IL-18 is proteolytically digested in the CF lung by neutrophil-derived serine proteases. The serine proteases NE, CG, and PR3 cleave common substrates, but our results have established that IL-18 is rapidly cleaved by NE, an effect more evident in *P. aeruginosa*-positive CF BALF compared with *P. aeruginosa*-negative CF samples. The latter observation can be explained at least in part by the ability of *Pseudomonas*-derived elastase to cleave IL-18 and also by greater numbers of neutrophils and, by corollary, greater levels of NE entering the microbe-infected CF lung. In addition, it has previously been shown that IL-8 expression is prolonged post *P. aeruginosa* stimulation (40), thus contributing to excessive neutrophil recruitment and NE burden within the CF airways. In support of our findings, NE inhibitors (A1AT, SLPI, elafin, and CMK) were able to inhibit the cleavage of IL-18, and purified NE was shown to rapidly cleave IL-18 in vitro. The biological consequence of NE-induced IL-18 degradation was investigated, and examination of IL-2 protein expression by Jurkat T cells revealed that NE abolished the IL-2–inducing ability of IL-18. Of particular interest, we have previously shown that IL-18 plays an important role in the regulation of IL-2 expression by pulmonary $CD4^+$ T lymphocytes in sarcoidosis and that epithelial cell lining fluid concentrations of IL-18 were significantly elevated (15). However, whereas the elastase-antiprotease balance within the sarcoidosis lung is balanced against elastolytic activity (41), supporting the high IL-18 levels within the sarcoidosis lung, within the CF lung, concentrations of NE are extremely high (in the range of 10^{-6} M) and directly correlate with the low levels of IL-18 recorded in this and in previous studies (5, 6).

IL-18 is probably best known for its IFN-γ–inducing ability and acts in synergy with IL-12 by inducing the production of IFN-γ by Th1 cells (42). By analogy, predisposition of patients with CF to microbial infection may in part be due to insufficient Th1 cell development and a result of low levels of IFN-γ as measured in this study; however, such inference clearly warrants further investigations. IL-18 has been implicated in immunity to various pathogens and is involved in protective immunity against pathogens including *Candida* and *Cryptococcus* (36) due to the described Th1-promoting function. *Staphylococcus aureus* and *P. aeruginosa* are the bacterial strains most frequently associated with pulmonary infection in patients with CF. Secretion of *Pseudomonas*-derived proteases, including the serine protease arginyl peptidase and three metalloproteases (pseudolysin, aeruginolysin and staphylolysin), have been recorded in sputum during bronchopulmonary exacerbations in bacterially infected CF individuals (43). These secreted proteases are capable of cleaving a number of inflammatory molecules including A1AT and SLPI and are thought to play a role in bacterial colonization. Our results demonstrate that in addition to neutrophil serine proteinases, IL-18 is also susceptible to the proteolytic activity of *Pseudomonas* proteases, as the cytokine is rapidly degraded in the presence of purified pseudolysin (PE). Nevertheless, although this study recognizes the potential of pseudolysin to degrade IL-18 in vitro, it must be pointed out that only serine and not metalloprotease inhibitors prevented IL-18 degradation within CF BALF. These results indicate that secreted pseudolysin may have a localized effect on cytokine proteolysis but that the more abundant neutrophil-derived proteases are mainly responsible for degradation of IL-18 within the CF lung.

The neutrophil numbers in BALF of patients with CF is estimated at least two orders of magnitude higher than patients with chronic bronchitis during a period of exacerbation (44). This neutrophilic inflammation is partially explained by the stability of IL-8 within the CF lung. The removal of HS and CS from lung tissue or silencing the enzyme arylsulfatase B that removes the 4-sulfate group from chondroitin-4-sulfate (45) decreases the binding of IL-8, suggesting that GAGs provide affinity-binding sites for IL-8 in lung tissue (31). In this study, the binding of GAGs to IL-8 and IL-18 was analyzed using surface plasmon resonance. In comparison with known methods (e.g., affinity chromatography and solid-phase binding assay), this method is advantageous in that no labeling of samples was required, and the experimental time was short (10 min per interaction). The results of this study are the first to show that IL-18 interacts with GAGs in a dose-dependent manner and afford additional information on cytokine interactions in real time. The results revealed dissimilar affinities of IL-8 and IL-18 for GAG oligosaccharides, with IL-8 illustrating significantly higher binding than IL-18. Most chemokines interact with GAGs via charge complementation, as they are basic proteins, whereas GAGs are acidic polymers. Certain GAG-protein interactions, including selenoprotein P (46) and PECAM-1 (47), are regulated by pH, and alterations of the pH can have a effect on the ability of proteins to bind to GAGs. In this study, lowering the pH was a relevant parameter to include within our Biacore-based analysis, as the pH in airways of CF individuals is known to be low (pH 5.8–6.6) (32, 33), and results revealed increased interaction of both IL-18 and IL-8 to GAGs at pH 6.3, possibly by increasing histidine protonation, and, in doing so, favoring the formation of electrostatic interactions with the negatively charged groups of GAGs. However, IL-8 greatly differs from IL-18 at both the sequence and structural level. Amino acids involved in IL-8–GAG binding have been identified as the basic amino acids His-18, Arg-60, Lys-64, Lys-67, and Arg68, located in the C-terminal helix and Lys20 in the proximal loop (48). The rank order of these residues' contributions to GAG binding is Lys-64, Arg-60, Lys-20, Lys-67, Arg-68, and His18. This sequence can be expressed as the Prosite pattern, H-x-K-x(39)-R-x(3)-K-x(2)-KR. IL-18 does not possess this motif but maintains a number of basic amino acids of electrostatic potential on the solvent accessible protein surface (34). Chemical modification of these surface arginine and lysine residues employing 2,3-butanedione and pyridoxal 5′-phosphate substantially reduced but did not abolish the interaction of IL-18. This latter result may suggest the involvement of an additional basic amino acid including the single histidine residue (His-145) of IL-18 in binding GAGs, thus supporting the observation of enhanced binding at low pH (Fig. 5*A*). A similar mechanism of interaction has been reported for GM-CSF, whereby protonation of two histidine residues in helix C of GM-CSF acts as a pH-dependent switch to control GAG interactions (49). Of particular interest, analogous to IL-18 and in contrast to IL-8, reduced CF BALF levels of GM-CSF have been reported compared with non-CF controls (50). Collectively, these results suggest that IL-18 can use electrostatic mechanisms to directly bind GAGs; however, further characterization of the IL-18–GAG interaction is currently under investigation to fully determine basic residues involved in GAG binding.

Significant changes occur in concentration and composition of GAGs at sites of inflammation. For example, a reduced level of HS

and increased CS has been observed in atherosclerosis (51). Increased sulphation and concentrations of GAGs (52, 53) have been found in BALF from children with CF, and secretion of HS (13), CS (26, 54), and HA (27, 55) is markedly increased in bronchial cells and CF tissues. Increased expression of GAGs (including HS) at the endothelium in CF may point to an exceptional characteristic of CF that contributes to increased stability of IL-8, thus leading to chronic neutrophil infiltration and inflammation (13). However, the amount of IL-8 bound to GAGs can also be influenced by other chemokines. Studies examining affinities of various chemokines for GAGs in vitro established a hierarchy in binding whereby RANTES > MCP-1 > IL-8 > MIP-1α (12). This result suggested that the amount of IL-8 bound to GAGs at sites of inflammation could be governed by the presence of other chemokines that may compete for low-affinity GAG binding sites. The major finding of this study demonstrated that the binding affinity of IL-8 to GAGs was greater than the affinity of IL-18. Accordingly, IL-8 possessed the ability not only to compete with IL-18, but, comparable to MIP-1α and RANTES, also displaced IL-18 from GAG-binding motifs. These observations suggest that IL-8 and IL-18 may interact with common GAG motifs, and the fact that IL-8 binds to GAGs with a higher affinity than IL-18 probably accounts for the displacement of the cytokine from GAGs in the presence of IL-8. Of note, molecular displacement from GAG anionic matrices is not restricted to IL-18. Indeed, it was previously suggested that insulin and lysozyme are capable of displacing β_2-microglobulin from binding brush border membranes (56). Additionally, the anti-inflammatory properties of lactoferrin during septicemia are related to its ability to competitively displace IL-8 binding from proteoglycans (57). Of particular interest is the fact that we have previously shown that proteolytic cathepsin activity in BALF from patients with CF is increased and results in degradation and loss of lactoferrin activity (58). Thus, depleted levels of lactoferrin may in part ameliorate the reported prolonged half-life of IL-8 within the CF lung (13).

The interaction of inflammatory molecules with GAGs was hypothesized to provide a mechanism for establishing chemokine gradients to provide directional signals for migrating cells (59). Additional roles for cytokine– and chemokine–GAG interactions include presentation of inflammatory molecules to respective receptors and protection from proteolytic degradation. Examples of cytokines bound to GAGs in a steady state include binding of HS to IFN-γ (60), limiting the extent of its C-terminal domain degradation (61), yet decreasing the plasma clearance and increasing the cytokine activity by up to 600% (62). In addition, a pharmacokinetic study substantiated the in vivo long-lasting characteristics of GAG-containing IFN-γ formulations (63). It has previously been shown that IL-8–GAG interactions facilitate binding of the cytokine to its specific receptor, prolong IL-8 activity, and protect it from proteolysis (13). IL-8 possesses several putative NE cleavage sites, and incubation of recombinant human IL-8 with NE results in loss of IL-8 chemotactic activity in a dose- and time-dependent manner (64). Nevertheless, IL-8 is present in CF lung samples despite high levels of NE, and, consequently, GAGs appear to represent an effective mechanism for maintaining the bioactivity of IL-8. The major finding of this study demonstrated that incubation with GAGs had a pronounced stabilizing effect on IL-18 and reduced both the rate and extent of NE-mediated proteolysis. It was proposed that GAGs, including HA administered by aerosolization, mitigate the action of elastases including NE and macrophage metalloelastase (65). However, a ratio of 10:1 and up to 100:1 of GAGs:enzyme did not inhibit the activity of NE in our in vitro studies. Moreover, competition between IL-8 and IL-18 for binding to GAGs exposed IL-18 to NE-mediated proteolysis, epitomizing the symptoms of low IL-18 levels in the CF inflammatory lung. Protection from elastolytic activity within the CF lung via binding to GAGs is not restricted to cytokines and chemokines, and, most recently, we have demonstrated that binding of the antimicrobial peptide LL-37 to GAGs protected the peptide form cathepsin D and, to a lesser extent, NE-mediated proteolysis (21).

The knowledge that GAG expression is altered in disease, along with evidence that cytokines are implicated in disease and interact with GAGs, suggests that interfering with this specific interaction may offer a treatment for many inflammatory diseases. A large body of work has been carried out in this area, and blocking interactions between IFN-γ and endothelial GAGs was suggested as a clinical method of immunosuppression (66), whereas mutation of the GAG binding site of RANTES has revealed a novel anti-inflammatory strategy (67). Moreover, anti-inflammatory properties of a cationic-derived peptide of mouse IFN-γ was shown to inhibit the GAG binding capacity of IL-8 (68). Such types of treatment may be useful in chronic airway inflammation to reduce levels of IL-8, but as these peptides are not specific, they may also inhibit binding of other cytokines such as IL-18. Although the research described in this study does not evaluate a therapeutic intervention, it does elucidate more fully the intricate interplay between GAGs and IL-8 and IL-18 and fully recognizes the potential of the GAG–cytokine interaction as a therapeutic target.

Acknowledgments

We thank Catherine M. Greene and Gudmundur Bergsson, Royal College of Surgeons in Ireland, for valuable discussion, Isabel Vega-Carrascal for assistance with illustrations, and Irene Oglesby, Royal College of Surgeons in Ireland, for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Stecenko, A. A., G. King, K. Torii, R. M. Breyer, R. Dworski, T. S. Blackwell, J. W. Christman, and K. L. Brigham. 2001. Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. *Inflammation* 25: 145–155.
2. Hartl, D., M. Griese, M. Kappler, G. Zissel, D. Reinhardt, C. Rebhan, D. J. Schendel, and S. Krauss-Etschmann. 2006. Pulmonary T(H)2 response in *Pseudomonas aeruginosa*-infected patients with cystic fibrosis. *J. Allergy Clin. Immunol.* 117: 204–211.
3. Richman-Eisenstat, J. B., P. G. Jorens, C. A. Hébert, I. Ueki, and J. A. Nadel. 1993. Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. *Am. J. Physiol.* 264: L413–L418.
4. Tabary, O., J. M. Zahm, J. Hinnrasky, J. P. Couetil, P. Cornillet, M. Guenounou, D. Gaillard, E. Puchelle, and J. Jacquot. 1998. Selective up-regulation of chemokine IL-8 expression in cystic fibrosis bronchial gland cells in vivo and in vitro. *Am. J. Pathol.* 153: 921–930.
5. Chan, E. D., H. S. Choi, C. Cool, F. J. Accurso, and G. Fantuzzi. 2002. Interleukin-18 expression in cystic fibrosis lungs. *Chest* 121(Suppl 3): 84S–85S.
6. Hauber, H. P., I. S. Beyer, A. Meyer, and A. Pforte. 2004. Decreased interleukin-18 expression in BAL cells and peripheral blood mononuclear cells in adult cystic fibrosis patients. *J. Cyst. Fibros.* 3: 129–131.
7. Muhlebach, M. S., and T. L. Noah. 2002. Endotoxin activity and inflammatory markers in the airways of young patients with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 165: 911–915.
8. Kettle, A. J., T. Chan, I. Osberg, R. Senthilmohan, A. L. Chapman, T. J. Mocatta, and J. S. Wagener. 2004. Myeloperoxidase and protein oxidation in the airways of young children with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 170: 1317–1323.
9. Kelly, E., C. M. Greene, and N. G. McElvaney. 2008. Targeting neutrophil elastase in cystic fibrosis. *Expert Opin. Ther. Targets* 12: 145–157.
10. Goger, B., Y. Halden, A. Rek, R. Mösl, D. Pye, J. Gallagher, and A. J. Kungl. 2002. Different affinities of glycosaminoglycan oligosaccharides for monomeric and dimeric interleukin-8: a model for chemokine regulation at inflammatory sites. *Biochemistry* 41: 1640–1646.
11. Marshall, L. J., L. S. Ramdin, T. Brooks, P. C. DPhil, and J. K. Shute. 2003. Plasminogen activator inhibitor-1 supports IL-8-mediated neutrophil transendothelial migration by inhibition of the constitutive shedding of endothelial IL-8/heparan sulfate/syndecan-1 complexes. *J. Immunol.* 171: 2057–2065.
12. Kuschert, G. S., F. Coulin, C. A. Power, A. E. Proudfoot, R. E. Hubbard, A. J. Hoogewerf, and T. N. Wells. 1999. Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry* 38: 12959–12968.
13. Solic, N., J. Wilson, S. J. Wilson, and J. K. Shute. 2005. Endothelial activation and increased heparan sulfate expression in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 172: 892–898.

14. Gracie, J. A., R. J. Forsey, W. L. Chan, A. Gilmour, B. P. Leung, M. R. Greer, K. Kennedy, R. Carter, X. Q. Wei, D. Xu, et al. 1999. A proinflammatory role for IL-18 in rheumatoid arthritis. *J. Clin. Invest.* 104: 1393–1401.
15. Greene, C. M., G. Meachery, C. C. Taggart, C. P. Rooney, R. Coakley, S. J. O'Neill, and N. G. McElvaney. 2000. Role of IL-18 in CD4+ T lymphocyte activation in sarcoidosis. *J. Immunol.* 165: 4718–4724.
16. Imaoka, H., T. Hoshino, S. Takei, T. Kinoshita, M. Okamoto, T. Kawayama, S. Kato, H. Iwasaki, K. Watanabe, and H. Aizawa. 2008. Interleukin-18 production and pulmonary function in COPD. *Eur. Respir. J.* 31: 287–297.
17. Takeda, K., H. Tsutsui, T. Yoshimoto, O. Adachi, N. Yoshida, T. Kishimoto, H. Okamura, K. Nakanishi, and S. Akira. 1998. Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8: 383–390.
18. Kim, S. H., M. Eisenstein, L. Reznikov, G. Fantuzzi, D. Novick, M. Rubinstein, and C. A. Dinarello. 2000. Structural requirements of six naturally occurring isoforms of the IL-18 binding protein to inhibit IL-18. *Proc. Natl. Acad. Sci. USA* 97: 1190–1195.
19. Robertson, S. E., J. D. Young, S. Kitson, A. Pitt, J. Evans, J. Roes, D. Karaoglu, L. Santora, T. Ghayur, F. Y. Liew, et al. 2006. Expression and alternative processing of IL-18 in human neutrophils. *Eur. J. Immunol.* 36: 722–731.
20. Wieland, C. W., B. Siegmund, G. Senaldi, M. L. Vasil, C. A. Dinarello, and G. Fantuzzi. 2002. Pulmonary inflammation induced by *Pseudomonas aeruginosa* lipopolysaccharide, phospholipase C, and exotoxin A: role of interferon regulatory factor 1. *Infect. Immun.* 70: 1352–1358.
21. Bergsson, G., E. P. Reeves, P. McNally, S. H. Chotirmall, C. M. Greene, P. Greally, P. Murphy, S. J. O'Neill, and N. G. McElvaney. 2009. LL-37 complexation with glycosaminoglycans in cystic fibrosis lungs inhibits antimicrobial activity, which can be restored by hypertonic saline. *J. Immunol.* 183: 543–551.
22. Kelly, D. M., C. M. Greene, G. Meachery, M. O'Mahony, P. M. Gallagher, C. C. Taggart, S. J. O'Neill, and N. G. McElvaney. 2005. Endotoxin up-regulates interleukin-18: potential role for gram-negative colonization in sarcoidosis. *Am. J. Respir. Crit. Care Med.* 172: 1299–1307.
23. Cozens, A. L., M. J. Yezzi, L. Chin, E. M. Simon, W. E. Finkbeiner, J. A. Wagner, and D. C. Gruenert. 1992. Characterization of immortal cystic fibrosis tracheobronchial gland epithelial cells. *Proc. Natl. Acad. Sci. USA* 89: 5171–5175.
24. Bergin, D. A., C. M. Greene, E. E. Sterchi, C. Kenna, P. Geraghty, A. Belaaouaj, A. Belaaouaj, C. C. Taggart, S. J. O'Neill, and N. G. McElvaney. 2008. Activation of the epidermal growth factor receptor (EGFR) by a novel metalloprotease pathway. [Published erratum appears in 2009 *J. Biol. Chem.* 284: 9624.] *J. Biol. Chem.* 283: 31736–31744.
25. Zeitlin, P. L., L. Lu, J. Rhim, G. Cutting, G. Stetten, K. A. Kieffer, R. Craig, and W. B. Guggino. 1991. A cystic fibrosis bronchial epithelial cell line: immortalization by adeno-12-SV40 infection. *Am. J. Respir. Cell Mol. Biol.* 4: 313–319.
26. Rahmoune, H., G. Lamblin, J. J. Lafitte, C. Galabert, M. Filliat, and P. Roussel. 1991. Chondroitin sulfate in sputum from patients with cystic fibrosis and chronic bronchitis. *Am. J. Respir. Cell Mol. Biol.* 5: 315–320.
27. Wyatt, H. A., A. Dhawan, P. Cheeseman, G. Mieli-Vergani, and J. F. Price. 2002. Serum hyaluronic acid concentrations are increased in cystic fibrosis patients with liver disease. *Arch. Dis. Child.* 86: 190–193.
28. Reeves, E. P., T. Ali, P. Leonard, S. Hearty, R. O'Kennedy, F. E. May, B. R. Westley, C. Josenhans, M. Rust, S. Suerbaum, A. Smith, B. Drumm, and M. Clyne. 2008. Helicobacter pylori lipopolysaccharide interacts with TFF1 in a pH-dependent manner. *Gastroenterology* 135: 2043–2054, 2054.e1–2.
29. Chen, S. S., and P. C. Engel. 1975. Dogfish M4 lactate dehydrogenase: reversible inactivation by pyridoxal 5′-phosphate and complete protection in complexes that mimic the active ternary complex. *Biochem. J.* 151: 447–449.
30. Swanborg, R. H. 1970. The effect of selective modification of tryptophan, lysine and arginine residues of basic brain protein on encephalitogenic activity. *J. Immunol.* 105: 865–871.
31. Frevert, C. W., M. G. Kinsella, C. Vathanaprida, R. B. Goodman, D. G. Baskin, A. Proudfoot, T. N. Wells, T. N. Wight, and T. R. Martin. 2003. Binding of interleukin-8 to heparan sulfate and chondroitin sulfate in lung tissue. *Am. J. Respir. Cell Mol. Biol.* 28: 464–472.
32. Tate, S., G. MacGregor, M. Davis, J. A. Innes, and A. P. Greening. 2002. Airways in cystic fibrosis are acidified: detection by exhaled breath condensate. *Thorax* 57: 926–929.
33. Newport, S., N. Amin, and A. J. Dozor. 2009. Exhaled breath condensate pH and ammonia in cystic fibrosis and response to treatment of acute pulmonary exacerbations. *Pediatr. Pulmonol.* 44: 866–872.
34. Kato, Z., J. Jee, H. Shikano, M. Mishima, I. Ohki, H. Ohnishi, A. Li, K. Hashimoto, E. Matsukuma, K. Omoya, et al. 2003. The structure and binding mode of interleukin-18. *Nat. Struct. Biol.* 10: 966–971.
35. Webb, L. M., M. U. Ehrengruber, I. Clark-Lewis, M. Baggiolini, and A. Rot. 1993. Binding to heparan sulfate or heparin enhances neutrophil responses to interleukin 8. *Proc. Natl. Acad. Sci. USA* 90: 7158–7162.
36. Kawakami, K., Y. Koguchi, M. H. Qureshi, Y. Kinjo, S. Yara, A. Miyazato, M. Kurimoto, K. Takeda, S. Akira, and A. Saito. 2000. Reduced host resistance and Th1 response to Cryptococcus neoformans in interleukin-18 deficient mice. *FEMS Microbiol. Lett.* 186: 121–126.
37. Khan, T. Z., J. S. Wagener, T. Bost, J. Martinez, F. J. Accurso, and D. W. Riches. 1995. Early pulmonary inflammation in infants with cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 151: 1075–1082.
38. Tirouvanziam, R., Y. Gernez, C. K. Conrad, R. B. Moss, I. Schrijver, C. E. Dunn, Z. A. Davies, L. A. Herzenberg, and L. A. Herzenberg. 2008. Profound functional and signaling changes in viable inflammatory neutrophils homing to cystic fibrosis airways. *Proc. Natl. Acad. Sci. USA* 105: 4335–4339.
39. Moss, R. B., Y. P. Hsu, and L. Olds. 2000. Cytokine dysregulation in activated cystic fibrosis (CF) peripheral lymphocytes. *Clin. Exp. Immunol.* 120: 518–525.
40. Joseph, T., D. Look, and T. Ferkol. 2005. NF-kappaB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with *Pseudomonas aeruginosa*. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 288: L471–L479.
41. Smith, S. F., A. Guz, G. H. Burton, R. Heaton, N. T. Cooke, and T. D. Tetley. 1988. The elastase: anti-elastase profile in lung lavage from sarcoidosis patients. *Sarcoidosis* 5: 31–37.
42. Okamura, H., H. Tsutsui, S. Kashiwamura, T. Yoshimoto, and K. Nakanishi. 1998. Interleukin-18: a novel cytokine that augments both innate and acquired immunity. *Adv. Immunol.* 70: 281–312.
43. Jaffar-Bandjee, M. C., A. Lazdunski, M. Bally, J. Carrère, J. P. Chazalette, and C. Galabert. 1995. Production of elastase, exotoxin A, and alkaline protease in sputa during pulmonary exacerbation of cystic fibrosis in patients chronically infected by *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* 33: 924–929.
44. Meyer, K. C., and A. Sharma. 1997. Regional variability of lung inflammation in cystic fibrosis. *Am. J. Respir. Crit. Care Med.* 156: 1536–1540.
45. Bhattacharyya, S., K. Solakyildirim, Z. Zhang, M. L. Chen, R. J. Linhardt, and J. K. Tobacman. 2009. Cell-bound IL-8 Increases in bronchial epithelial cells following arylsulfatase B silencing. *Am. J. Respir. Cell Mol. Biol.* doi:10.1165/rcmb.2008-0482OC
46. Arteel, G. E., S. Franken, J. Kappler, and H. Sies. 2000. Binding of selenoprotein P to heparin: characterization with surface plasmon resonance. *Biol. Chem.* 381: 265–268.
47. Gandhi, N. S., D. R. Coombe, and R. L. Mancera. 2008. Platelet endothelial cell adhesion molecule 1 (PECAM-1) and its interactions with glycosaminoglycans: 1. Molecular modeling studies. *Biochemistry* 47: 4851–4862.
48. Krieger, E., E. Geretti, B. Brandner, B. Goger, T. N. Wells, and A. J. Kungl. 2004. A structural and dynamic model for the interaction of interleukin-8 and glycosaminoglycans: support from isothermal fluorescence titrations. *Proteins* 54: 768–775.
49. Sebollela, A., T. C. Cagliari, G. S. Limaverde, A. Chapeaurouge, M. H. Sorgine, T. Coelho-Sampaio, C. H. Ramos, and S. T. Ferreira. 2005. Heparin-binding sites in granulocyte-macrophage colony-stimulating factor. Localization and regulation by histidine ionization. *J. Biol. Chem.* 280: 31949–31956.
50. Alexis, N. E., M. S. Muhlebach, D. B. Peden, and T. L. Noah. 2006. Attenuation of host defense function of lung phagocytes in young cystic fibrosis patients. *J. Cyst. Fibros.* 5: 17–25.
51. Marquezini, M. V., C. M. Strunz, L. A. Dallan, and O. M. Toledo. 1995. Glycosaminoglycan distribution in atherosclerotic saphenous vein grafts. *Cardiology* 86: 143–146.
52. Bhaskar, K. R., B. S. Turner, S. A. Grubman, D. M. Jefferson, and J. T. LaMont. 1998. Dysregulation of proteoglycan production by intrahepatic biliary epithelial cells bearing defective (δ-f508) cystic fibrosis transmembrane conductance regulator. *Hepatology* 27: 7–14.
53. Hilliard, T. N., N. Regamey, J. K. Shute, A. G. Nicholson, E. W. Alton, A. Bush, and J. C. Davies. 2007. Airway remodelling in children with cystic fibrosis. *Thorax* 62: 1074–1080.
54. Khatri, I. A., K. R. Bhaskar, J. T. Lamont, S. U. Sajjan, C. K. Ho, and J. Forstner. 2003. Effect of chondroitinase ABC on purulent sputum from cystic fibrosis and other patients. *Pediatr. Res.* 53: 619–627.
55. Sahu, S. C. 1980. Hyaluronic acid. An indicator of pathological conditions of human lungs? *Inflammation* 4: 107–112.
56. Dreisbach, A. W., and V. Batuman. 1994. Low-molecular-weight protein competition for binding sites on renal brush border membranes. *Ren. Physiol. Biochem.* 17: 287–293.
57. Elass, E., M. Masson, J. Mazurier, and D. Legrand. 2002. Lactoferrin inhibits the lipopolysaccharide-induced expression and proteoglycan-binding ability of interleukin-8 in human endothelial cells. *Infect. Immun.* 70: 1860–1866.
58. Rogan, M. P., C. C. Taggart, C. M. Greene, P. G. Murphy, S. J. O'Neill, and N. G. McElvaney. 2004. Loss of microbicidal activity and increased formation of biofilm due to decreased lactoferrin activity in patients with cystic fibrosis. *J. Infect. Dis.* 190: 1245–1253.
59. Proudfoot, A. E., T. M. Handel, Z. Johnson, E. K. Lau, P. LiWang, I. Clark-Lewis, F. Borlat, T. N. Wells, and M. H. Kosco-Vilbois. 2003. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc. Natl. Acad. Sci. USA* 100: 1885–1890.
60. Sadir, R., E. Forest, and H. Lortat-Jacob. 1998. The heparan sulfate binding sequence of interferon-γ increased the on rate of the interferon-γ-interferon-γ receptor complex formation. *J. Biol. Chem.* 273: 10919–10925.
61. Lortat-Jacob, H., and J. A. Grimaud. 1991. Interferon-γ binds to heparan sulfate by a cluster of amino acids located in the C-terminal part of the molecule. *FEBS Lett.* 280: 152–154.
62. Lortat-Jacob, H., F. Baltzer, and J. A. Grimaud. 1996. Heparin decreases the blood clearance of interferon-γ and increases its activity by limiting the processing of its carboxyl-terminal sequence. *J. Biol. Chem.* 271: 16139–16143.
63. Ito, Y., A. Saeki, K. Shiroyama, N. Sugioka, and K. Takada. 2008. Percutaneous absorption of interferon-α by self-dissolving micropiles. *J. Drug Target.* 16: 243–249.
64. Leavell, K. J., M. W. Peterson, and T. J. Gross. 1997. Human neutrophil elastase abolishes interleukin-8 chemotactic activity. *J. Leukoc. Biol.* 61: 361–366.
65. Cantor, J. O., J. M. Cerreta, G. Armand, and G. M. Turino. 1998. Aerosolized hyaluronic acid decreases alveolar injury induced by human neutrophil elastase. *Proc. Soc. Exp. Biol. Med.* 217: 471–475.
66. Douglas, M. S., D. A. Rix, J. H. Dark, D. Talbot, and J. A. Kirby. 1997. Examination of the mechanism by which heparin antagonizes activation of a model endothelium by interferon-γ (IFN-γ). *Clin. Exp. Immunol.* 107: 578–584.
67. Baltus, T., K. S. Weber, Z. Johnson, A. E. Proudfoot, and C. Weber. 2003. Oligomerization of RANTES is required for CCR1-mediated arrest but not CCR5-mediated transmigration of leukocytes on inflamed endothelium. *Blood* 102: 1985–1988.
68. Cripps, J. G., F. A. Crespo, P. Romanovskis, A. F. Spatola, and R. Fernández-Botrán. 2005. Modulation of acute inflammation by targeting glycosaminoglycan-cytokine interactions. *Int. Immunopharmacol.* 5: 1622–1632.