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

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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 test 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-18BPs 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
explanation it has also been speculated that *Pseudomonas aerugi-
osa*-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 in-
flammatory 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 pa-
rental consent was obtained prior to bronchoscopy. All procedures were
continued 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
years, forced expiratory volume in 1 s (FEV1) 64.65 ± 6.84% (mean ± SE)], and 17 were noncolonized [average age 7.78 ± 2.11 years, FEV1 61.00 ± 8.67% (mean ± SE)]. 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 51.5 years (mean ± SE), FEV1 was 52.25 ± 8.27%. BALF was centrifuged at 2000 × g for 10 min and the super-
nant aliquoted and stored at −80°C. The BALF cell pellet was re-
suspended 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. Cy-
tokine-specific Abs were employed: mouse IgG1, antihuman IL-18, mouse
IgG1, antihuman IL-18BPA, mouse IgG2a, antihuman IL-8, and mouse IgG2a,
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, Hertford-
shire, 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 (Invitro-
gen, CA, U.S.A.). For Western blot analysis, proteins were transferred to	polyvinylidene difluoride membrane at 100 mA for 1 h using a semidry
electrophoresis and Western blot analysis as described above.

**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 centri-
fugation (Axis-Shield, Cambridge, U.K.). The mononuclear cell in-
terphase was removed and washed with HBSS (Life Technologies).
Subsequently, monocytes were purified to 97% purity using the EasySep
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 × 10⁴) 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, gel electrophoresis and Western blot analysis, as already described.

For measurement of IL-18 secretion by monocytes, cell culture condi-
tions and protocol for cell activation were as previously described (22).
In brief, monocytes (5 × 10⁶) 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 CFBE410± (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
employed for the current and previous studies described above. RT-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′-CACGCGCTT-
TGACCAGCA-3′, 5′-CAAGAGAATGCTCCAGTGC-3′; β-actin: 5′-
GGTACATGGTGCCG-3′, 5′-GCCGGGAAATGTCGCG-3′; caspase 1: 5′-
GTTTCTTGGGATCACCAG-3′, 5′-TAATGTCCCTGGAAGA-
GAGG-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 × 10⁵ 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 superna-
tants 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 µM).

**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 in-
hibiting 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 µM benzamidine (Benz), 0.15 mM
N-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), 0.5 mM
N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), 20 µM N-
(Methoxysuccinyl)-Ala-Ala-Pro-Val-chloromethyl ketone (CMK), 10 µM
sobyten trypsin inhibitor (SBTI), 20 µM orthophenanthroline (OP), 1 µM
recombinant human elafin (Proteo Biotech AG, Kiel, Germany), 1 µM
0.1-antichymotrypsin (ACT), 1 µM α-1 antitrypsin (A1AT), 10 µM
EDTA, 25 µM E6.1 T cells, cell culturing conditions and concentrations of cell activators
were as previously described (15). Cells (5 × 10³ 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 superna-
tants 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 µM).
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 protease 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-(Methoxy-succinyl)-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 CMS 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 days (interday), and binding responses (responsivity 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 Na2CO3 and 35 mM NaHCO3, [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 GAGs exhibited a ratio of 1:10 (w/w) for each GAG prior to proteases exposure.

**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 chromatographic 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 500 ng) 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 (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 PBS. Adsorption of IL-18 to GAGs exhibited a ratio of 1:10 (w/w) for each GAG prior to proteases exposure.

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

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. 1F).

**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-18BPs and IFN-γ levels in BALF of control (*n* = 5) and CF (*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 *r* 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. 2A). 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. 2B) 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 samples was a result of defective processing of pro–IL-18 was normalized in relation to the GADPH loading control (Fig. 2B) with equal expression detected in all three human airway cell lines tested. 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. 3A, 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. 3B, 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.

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. 3C, 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. 4A, 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. 4B, 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 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. 4C). 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. 4D, 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. 4D), 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. 4E, 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 (10⁻⁵ M purified protease for 2 h at 37˚C) showed that elastase activity increased almost linearly, with an approximate 53.06% reduction in detectable levels of the cytokine in P. aeruginosa-colonized CF BALF.

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. 4F). IL-2 secretion was measured in cell culture
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 peflabol (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/mm² and 4.2 ng/mm² 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. 5A 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 5.29 to 6.15, 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. 5B 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. 5C, 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
The absence of GAGs was investigated. A time course experiment was performed by incubating RhIL-18 (100 ng) with NE (10^{-12} - 10^{-5} 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. 5D 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. 5E). 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. 6A). 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. 6A, 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. 6B). 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.
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/cm² 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. 6B).

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/cm² detected. In addition, IL-18 was abolished to undetectable levels when IL-8 (25 ng) was added 1 h after the addition of IL-18 (6 ng) (Fig. 6B). 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. 6C). 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. Summary 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. 6D), 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. 6E, 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 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.
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, provisionally 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.

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, provisionally 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 dysregulation 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-18BPα (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 been previously 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, eflavin, 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 inflammation and that epithelial cell lining fluid concentrations of 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 arginine peptidase and three metalloproteases (pseudolysin, aeruginolysin and staphylolysin), have been recorded in spum 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 neutrophil 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 aroylsulfatase 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-67, 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 His-18. 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. 5A). A similar mechanism of interaction has been reported for GM-CSF, whereby protonation of two histidine 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 His-18. 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. 5A). A similar mechanism of interaction has been reported for GM-CSF, whereby protonation of two histidine 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 His-18. 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.
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 bound 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 β2-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.

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