



Production of the potent neutrophil chemokine, growth-related protein α (GRO α), is not elevated in cystic fibrosis children

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Progressive neutrophil-mediated lung damage causes much of the morbidity and mortality in cystic fibrosis (CF). Neutrophil chemoattractants implicated in CF include interleukin (IL)-8, tumour necrosis factor (TNF α) and leukotriene (LT)B₄, but growth-related protein α (GRO α), a highly potent neutrophil chemokine, has not been investigated. Atopic status has been considered to contribute to the marked heterogeneity of pulmonary disease in CF. We hypothesized that GRO α may be produced in biologically-significant amounts in the CF lung, and that enhanced production of GRO α , IL-8 or LTB₄ may contribute to the poorer lung function seen in atopic CF patients compared to non-atopic CF patients. GRO α , IL-8 and LTB₄ levels in the sputum of atopic and non-atopic CF patients were assessed by immunoassays, and GRO α and IL-8 levels were also assessed in the plasma of CF patients and normal controls. As expected, there were high levels of IL-8 and LTB₄ in most CF sputum samples, and IL-8 levels were higher in CF plasma than in control plasma ($P=0.02$). In contrast, GRO α was undetectable ($<5 \text{ pg ml}^{-1}$) in the sputum of 21 out of 25 CF patients, with low levels (range 144–825 pg ml^{-1}) in the remainder, and median levels of GRO α in CF plasma (33 pg ml^{-1} , $n=24$) were not significantly different from controls (34 pg ml^{-1} , $n=25$). Lung function [forced expiratory volume in 1 sec (FEV₁) and forced vital capacity (FVC)] was significantly poorer in atopic CF compared to non-atopic CF patients ($P<0.02$), but sputum levels of GRO α , IL-8 and LTB₄ were not different between the subgroups. Our results suggest that unlike LTB₄ and IL-8, GRO α does not contribute to neutrophilic inflammation in the CF lung, and other factors must determine the impaired lung function observed in atopic CF patients. These results may have important implications in the development of chemokine receptor antagonists as novel anti-inflammatory agents in CF.

Key words: cystic fibrosis; chemokine; GRO α ; atopy.

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Introduction

Unrestrained local inflammation contributes significantly to progressive lung damage in cystic fibrosis (CF), mediated primarily by toxic products of neutrophils, especially oxygen free radicals and elastase. The mobilization of neutrophils from the circulation into the lungs is a complex series of events largely affected by adhesion molecule interactions and the production of chemoattractant lipids and cytokines, including leukotriene (LT)B₄, tumour necrosis factor (TNF α), interleukin (IL)-1, neutrophil-activating peptide (ENA-78) and IL-8. LTB₄ and IL-8 have been specifically implicated in airway inflammation in the CF lung (1,2). Growth-related protein alpha (GRO α) is a chemokine of the α sub-family and is structurally related

to IL-8, but with even more potent neutrophil chemoattractant activity which is exerted via the IL-8 receptor (3). Previously described as growth-related oncogene product α and melanocyte growth-stimulating activity (MGSA), GRO α is released in response to IL-1, TNF α and other pro-inflammatory stimuli acting on melanoma and other malignant cells, and also from normal cells including monocyte-macrophages, platelets and endothelial cells. GRO α is implicated in conditions characterized by chronic neutrophilic inflammation, such as psoriasis and rheumatoid arthritis (4,5), and levels of GRO α 3- to 7-fold higher than those of IL-8 have been found in bronchoalveolar lavage (BAL) fluid from patients with bacterial pneumonia, adult respiratory distress syndrome and *Pneumocystis* pneumonia (6). However, the role of GRO α in CF has never been explored. We hypothesized that GRO α may be important in perpetuating neutrophilic inflammation in the CF lung.

The severity of lung disease varies enormously in patients with CF, even between siblings and between non-related patients with the same CFTR genotype. It is likely that

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genetic and environmental factors interact to cause this heterogeneity, and many of these factors are unclear. While neutrophilic inflammation usually occurs in response to infection, high levels of inflammatory markers have also been demonstrated in bronchoalveolar lavage fluid (BALF) of CF infants for whom clinical and microbiological evidence of associated infection is unconvincing (7,8). We have previously described the adverse influence of positive atopic status on lung function in CF, and its association with increased production of bronchoconstrictor cysteinyl-leukotrienes, compared to non-atopic CF patients (9,10,11). We hypothesized that atopic CF patients may also over-produce the neutrophil chemotaxin LTB₄. Since LTB₄ has immunomodulatory actions in regulating cytokine expression by mononuclear cells (12,13) a putative overproduction of LTB₄ may lead to enhanced neutrophil migration in atopic CF patients compared to non-atopic CF patients not only by direct chemotaxis, but also indirectly by inducing expression of IL-8 and/or GRO α from other cells.

We aimed therefore, firstly to determine whether GRO α is present in the sputum and plasma of CF patients at biologically-significant levels, and secondly, to relate atopic status to sputum levels of IL-8, GRO α and LTB₄, and to impairment of lung function.

Methods

PATIENT RECRUITMENT AND SAMPLE COLLECTION

Ethical approval was obtained from King's Healthcare and Great Ormond Street Hospital for Children, Research Ethics Committees. Samples of sputum ($n=25$) and plasma ($n=24$) were collected from CF patients recruited at these regional paediatric clinics. The diagnosis of CF had been confirmed by sweat sodium levels above 60 mmol l⁻¹ and/or by the presence of two known CF gene mutations. The atopic CF patients were defined by one or more positive skin reactions (≥ 3 mm compared to control) to a panel of eight common allergens (cat and dog hair, house dust mite, house dust, mixed moulds, tree and grass pollens, mixed feathers). Mean (\pm SD) ages of the atopic CF patients (13.7 ± 3.3 yr) and non-atopic CF patients (12.2 ± 3.6 yr) were not significantly different. Lung function was assessed from spirometric measurements of forced expiratory volume (FEV₁) and forced vital capacity (FVC), expressed as a percentage of those predicted for age, sex and height (14). Lung function was used to detect differences between atopic and non-atopic CF patients and was also correlated with sputum levels of LTB₄, IL-8 and GRO α . Subjects were excluded by the use of corticosteroids, theophylline or non-steroidal anti-inflammatory drugs within the preceding four weeks. Control plasma was obtained from 25 healthy children (9.33 ± 3.1 yr) having routine blood sampling prior to minor surgical procedures. Seven of the control subjects had positive skin prick tests, and four other controls, with negative skin tests, were considered to be atopic on the basis of an elevated serum total IgE (with levels of 806, 863 and two > 1000 IU/ml respectively).

Where possible sputum was obtained from CF patients when they were clinically stable. However four subjects were only able to expectorate sputum whilst on IV antibiotics for an exacerbation. Their sputum IL-8 and LTB₄ levels were no greater than the other patients and they had undetectable GRO α levels. The volume of sputum available for analysis from the remainder of the subjects was limited and tests for GRO α and IL-8 were prioritized. Sputum samples were collected onto ice and stored at -20°C . Initial mucolysis was achieved using an equal volume of 100% N-acetylcysteine. Samples were then liquefied by homogenization and debris removed by low-speed centrifugation. The supernatant was divided into separate aliquots for analysis of LTB₄ and cytokines. Blood anti-coagulated with EDTA was collected onto ice, centrifuged within 30 min and the plasma stored at -20°C .

HPLC/RADIOIMMUNOASSAY OF LTB₄ IN SPUTUM

Liquefied sputum samples from 14 of the 25 CF patients (eight atopic, six non-atopic) were available for LTB₄ analysis. Tritium-labelled LTB₄ (4nCi; Amersham, U.K.) was added to allow correction for losses of endogenous LTB₄ during purification. The sputum was treated with four volumes of methanol at 4°C for 30 min, and debris removed by centrifugation at 1000g for 20 min. The supernatant was evaporated to dryness and reconstituted in distilled water. LTB₄ was further purified using Sep-Pak octadecylsilane cartridges (Millipore, Watford, U.K.) and high performance liquid chromatography (HPLC) with reverse-phase C18 columns and a methanol/water mobile phase as described previously (1). Radioimmunoassay (RIA) for LTB₄ was then performed as described (1) using a highly specific rabbit polyclonal antibody (gift of Dr A.W. Ford-Hutchinson, Merck-Frosst, Canada) with a minimum detection limit of approximately 20 pg ml⁻¹.

ENZYME IMMUNOASSAYS OF IL-8 AND GRO α IN SPUTUM AND PLASMA

Interleukin-8 and GRO α were measured in liquefied sputum ($n=25$) or plasma by specific and sensitive Quantikine enzyme linked immunosorbent assays (ELISA) (R&D Systems, Abingdon, U.K.) in accordance with the manufacturer's instructions. Samples were added to microtitre wells precoated with monoclonal antibody to the human recombinant cytokine and incubated for 2 h at room temperature. Wells were treated with a secondary antibody conjugated to horseradish peroxidase and bound cytokine visualized with tetramethylbenzidine chromogen and hydrogen peroxide by spectrophotometry at 450 nm. Assays were calibrated from a standard curve constructed using recombinant human cytokine over the range 0–6000 pg ml⁻¹ for IL-8 and 0–1000 pg ml⁻¹ for GRO α . Minimum detection limits were consistent with those determined by the kit manufacturer (< 18.1 pg ml⁻¹ for IL-8 and < 5 pg ml⁻¹ for GRO α).

STATISTICAL ANALYSIS

The normal range of GRO α levels in sputum has not been fully established, which precluded power calculations, but the numbers of subjects in each group were based on previous published CF studies showing significant differences in cytokine and leukotriene levels (1,2,9). LTB $_4$, IL-8 and GRO α levels were non-normally distributed and group comparisons were performed by Mann–Whitney *U*-test for non-parametric data. Results are described as median and range. FEV $_1$ and FVC were normally distributed and group comparisons were performed by unpaired Student's *t*-test. These results are described as mean \pm SD. Analyses were performed using the Minitab statistical package and $P \leq 0.05$ was considered significant.

Results

LUNG FUNCTION

FEV $_1$ was significantly lower in atopic CF patients ($41.2 \pm 14.7\%$ predicted, $n=12$) than in non-atopic CF patients ($59.4 \pm 16.6\%$; $n=13$, $P=0.008$). FVC was also significantly lower in the atopic CF group ($54.5 \pm 19.7\%$) than in the non-atopic CF group ($72 \pm 20.9\%$, $P=0.02$). The relatively poor lung function in both CF subgroups may reflect their selection as reliable producers of sputum samples.

SPUTUM IL-8, LTB $_4$ AND GRO α CONCENTRATIONS

Enzyme immunoassay showed that IL-8 was present at detectable levels (> 18.1 pg ml $^{-1}$) in the sputum of nine out of 12 atopic CF patients and in 12 out of 13 non-atopic CF patients [Fig. 1(a)]. IL-8 concentrations were not significantly different in the atopic CF group (404 pg ml $^{-1}$, range < 18.1 –7836) compared to the non-atopic CF group (738 pg ml $^{-1}$, range < 18.1 –9008; $P=0.18$). Sputum also contained high levels of LTB $_4$, with concentrations in atopic CF patients (723 pg ml $^{-1}$, range 301–1199, $n=8$) not significantly different from those in non-atopic CF patients (1020 pg ml $^{-1}$, range 571–3298, $n=6$; $P=0.3$) [Fig. 1(b)]. In contrast, GRO α levels were undetectable (< 5 pg ml $^{-1}$) in 10 out of 12 atopic CF patients and in 11 out of 13 non-atopic CF patients [Fig. 1(c)]. The remaining two patients in each CF subgroup had low GRO α levels ranging from 144 to 824 pg ml $^{-1}$. Levels of IL-8, LTB $_4$ and GRO α in CF sputum showed no significant correlations with FEV $_1$ or FVC ($r^2 \leq 0.1$, $P \geq 0.1$).

PLASMA IL-8 AND GRO α CONCENTRATIONS

The CF group comprised 24 subjects as additional plasma samples were obtained from two atopic patients who were unable to produce sputum, and three of the non-atopic

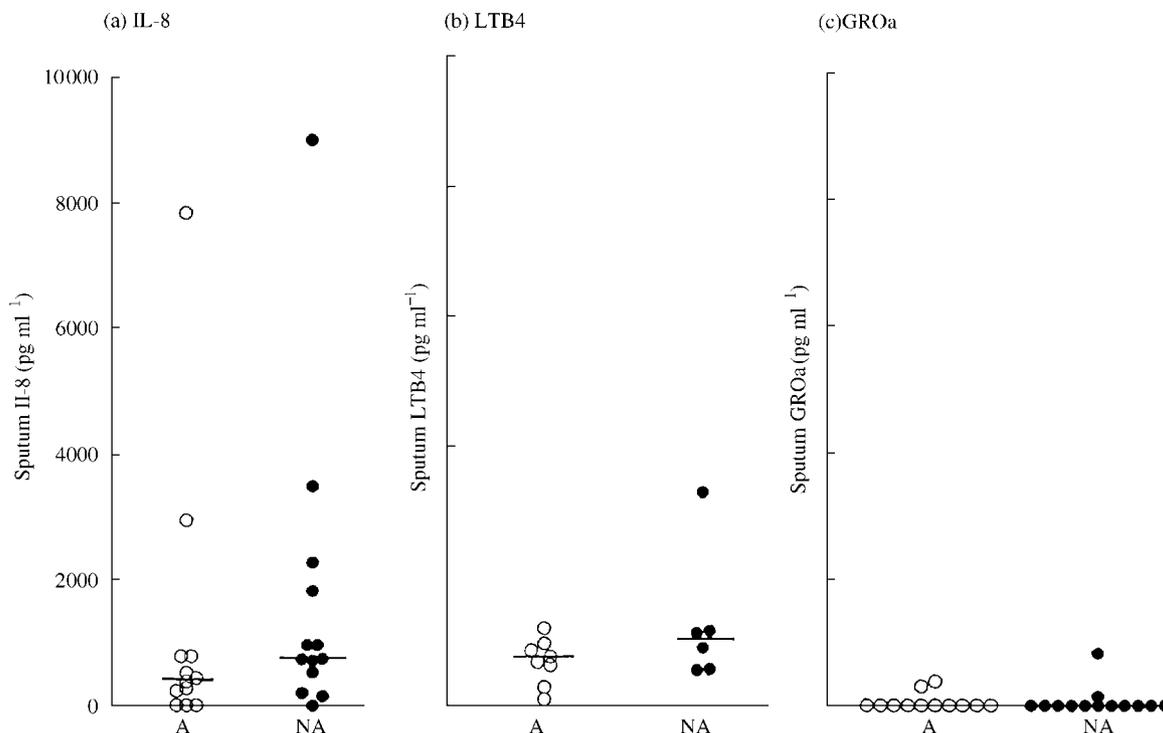


FIG. 1. Sputum levels of interleukin-8 (left), leukotriene B $_4$ (centre) and GRO α (right) in atopic (A) and non-atopic (NA) children with cystic fibrosis (CF). Median values are shown as horizontal bars. In contrast to IL-8 and LTB $_4$, GRO α was not detectable (< 5 pg ml $^{-1}$) in 10 out of 12 atopic CF patients and in 11 out of 13 non-atopic CF patients. There were no significant differences between the atopic and non-atopic CF patients in sputum levels of IL-8, LTB $_4$ or GRO α .

subjects who had produced sputum were unwilling to provide a blood sample. The apparent division of control subjects into two groups according to their plasma IL-8 level [Fig. 2(a)] is considered artificial and the undetectable levels are likely to form a continuum. No normal subject had any evidence of an acute illness and the variation in values of IL-8 is reasoned to reflect the normal range in children. Enzyme immunoassay showed that median levels of IL-8 were significantly higher in the plasma of CF patients (20 pg ml⁻¹, range <18.1–58.6, $n=24$) than in the plasma of normal subjects (18.3 pg ml⁻¹, range <18.1–24.1, $n=25$; $P=0.02$) [Fig. 2(a)]. IL-8 concentrations were not significantly different between the atopic CF and non-atopic CF subgroups. Median levels of GRO α in the plasma of CF patients (33 pg ml⁻¹, range <5–198, $n=24$) were not significantly different from those in normal subjects (34 pg ml⁻¹, range <5–64, $n=25$; $P>0.5$), and there were also no significant difference in plasma GRO α levels between the atopic CF and non-atopic CF subgroups [Fig. 2(b)]. Where paired samples were available from CF subjects IL-8 concentrations in plasma showed a correlation with those in sputum ($r^2=0.35$, $P=0.01$). There was no correlation between plasma IL-8 (or GRO α) levels and lung function.

Discussion

Characterization of the profile of factors that induce neutrophil influx and activation in the CF lung may lead to novel specific anti-inflammatory therapies. Growth-related protein α (GRO α) is a highly potent member of the α sub-family of neutrophil chemokines, and is implicated in neutrophilic inflammation in other lung diseases (6) and in psoriasis and rheumatoid arthritis (4,5). This study is the first to examine whether GRO α may also contribute to the intense neutrophilia that leads to chronic lung damage in cystic fibrosis. The study confirmed the findings of previous studies (1,2) that two other neutrophil chemotaxins (IL-8 and LTB₄) are present in CF sputum at biologically-significant concentrations. It also confirmed elevated levels of IL-8 in the plasma of CF patients compared to normal subjects. However, specific enzyme immunoassays failed to detect GRO α in 21 out of 25 CF sputum samples, and GRO α was present only at low levels in the remaining four patients. Furthermore, GRO α levels were not different in plasma from CF and normal subjects. The results suggest that GRO α does not contribute to neutrophil infiltration or activation in the CF lung.

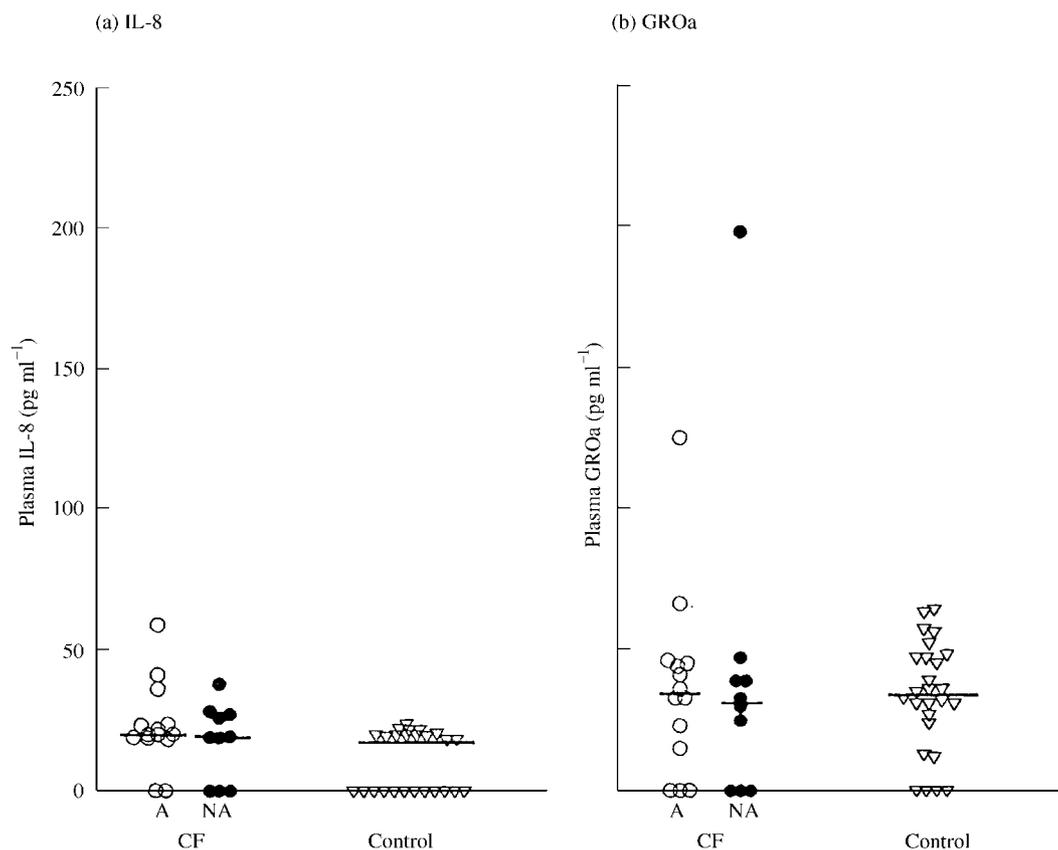


FIG. 2. Plasma levels of (a) IL-8 and (b) GRO α in atopic (A) and non-atopic (NA) cystic fibrosis (CF) subjects and controls. Median values are shown as horizontal bars. In the CF group as a whole, IL-8 levels were significantly higher than in controls ($P=0.02$), but GRO α levels were not significantly different from controls. There were no differences between the atopic and non-atopic CF subgroups in plasma levels of IL-8 or GRO α .

For a number of reasons, the lack of GRO α in CF sputum is likely to reflect a true deficiency due to impaired local production, and not a failure of detection: firstly, group sizes of 24–25 subjects provided a high level of statistical power to discriminate putative differences in plasma GRO α levels, and to detect GRO α in sputum in a small proportion of CF patients. Secondly, the sensitivity of the enzyme immunoassay is sufficient to detect GRO α at concentrations one to three orders of magnitude lower than those at which it exerts its biological effects on neutrophils and other cells (3). Thirdly, cytokine levels measured in CF sputum vary widely from study to study (15), but we investigated GRO α using techniques similar to those in a study in which significant GRO α levels were detected in BAL fluid from patients with respiratory diseases other than CF (6). Fourthly, the structurally-related α -family chemokine IL-8 was readily detected at nanomolar concentrations in the same sputum samples in which GRO α levels were negligible; both IL-8 and GRO α are likely to be degraded within the airway by non-specific proteases and there is no reason to suspect preferential degradation or dispersal of GRO α . Indeed, other studies show good correlations between cytokine concentrations in BAL fluid or sputum (2). Fifthly, although sputum is a notoriously heterogeneous medium, liquefaction of sputum using the mucolytic dithiothreitol (DTT) does not appear to affect chemokine immunoreactivity (16,17). Sixthly, plasma levels of GRO α are likely to represent overspill of macrophage-derived GRO α from the lung and elsewhere, and GRO α was readily detected using the same methodology in the majority of plasma samples from both CF patients and normal controls.

Since GRO α and IL-8 are produced *in vitro* by a similar range of cell-types (macrophages, endothelium) following stimulation by the same pro-inflammatory cytokines (IL-1, TNF α), it remains unclear why high IL-8 levels were seen in CF sputum in the absence of significant GRO α . Further work is required on the profile of stimuli in CF sputum and their effects on IL-8 and GRO α gene transcription. However, if GRO α had been present in CF sputum at similar molar levels to those of IL-8, the greater potency of GRO α (3) would mean that GRO α , not IL-8 would be the predominant ligand at the shared IL-8 receptor on neutrophils and other target cells. Our finding of negligible levels of GRO α in CF sputum suggests that novel anti-inflammatory agents need only block the binding of IL-8 to the shared receptor to reduce neutrophil migration in the CF lung.

The second aim of the study was to investigate whether sputum levels of neutrophil chemotaxins are different in atopic and non-atopic CF patients. Our previous studies showed that sputum cysteinyl-leukotriene (cys-LT) levels correlate with impairment of lung function in CF (10), and that atopic CF patients have worse lung function and higher systemic cys-LT production (assessed by urinary LTB $_4$ levels) than non-atopic CF children (9,11). Since cys-LTs and LTB $_4$ are formed from a common substrate by the 5-lipoxygenase pathway, which appears to be upregulated in atopy (18), and since LTB $_4$ is found in CF sputum (1), we hypothesized that there may be excessive LTB $_4$ production

in the atopic CF lung which could contribute to poorer lung function by inducing neutrophil migration, either by direct chemotaxis, or indirectly by enhancing expression of neutrophil chemokines such as IL-8 or GRO α (12,13). Although the atopic CF subgroup did show significantly lower FEV $_1$ and FVC values than their non-atopic counterparts, our hypothesis is contradicted by the lack of significant differences between the subgroups in sputum levels of LTB $_4$, IL-8 and GRO α , and in plasma levels of IL-8 and GRO α , and by the lack of correlations with lung function. The measurements of lung function used in the study (FEV $_1$ and FVC) may better reflect the consequences of over-production of the potent bronchoconstrictor cys-LTs. The atopic CF subgroup nevertheless remain an important subgroup who may gain additional benefit from specific anti-inflammatory therapy in the future, particularly with cys-LT receptor antagonists. Clinical trials with a 5-lipoxygenase inhibitor would help to define the contribution of LTB $_4$ to neutrophilic inflammation in CF.

In summary, the study found no evidence that the potent neutrophil chemokine GRO α is over-produced in the CF lung, despite confirming over-production of other neutrophil chemoattractants (IL-8, LTB $_4$) that are largely derived from similar cellular sources. Such a lack of GRO α at the site of intense neutrophil inflammatory activity may even contribute to the inability of neutrophils in the CF lung to eradicate respiratory infections. This finding may nevertheless promote the development of novel therapies based upon antagonism of the restricted range of neutrophil chemotaxins with a probable pathophysiological role in the CF lung, especially LTB $_4$ and/or IL-8.

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