Knockdown of interleukin-8 in airway epithelial cells

Abstract

Introduction: Cystic fibrosis (CF) is a neutrophil-dominated lung disease. The neutrophil chemokine interleukin-8 (IL-8) is present at higher than normal levels in the lungs of individuals with CF and is a key factor responsible for neutrophil infiltration into the lungs. Lipopolysaccharide (LPS) expressed by *Pseudomonas aeruginosa* in the CF lung can stimulate IL-8 expression by airway epithelial cells. Inhibiting the expression of IL-8 using small-interfering RNA (siRNA) represents a potential gene therapy approach for CF. Here, the efficacy of an IL-8-directed siRNA at inhibiting IL-8 gene and protein expression is tested in basal and LPS-stimulated human bronchial epithelial cells.

Methods: 16HBE14O$^-$ cells were transfected with either scrambled, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or IL-8-specific siRNAs. RNA was then isolated and used in quantitative real-time PCR to measure GAPDH, IL-8 or beta-actin (housekeeping) gene expression. Lastly, IL-8 protein production was quantified in cell supernatants by IL-8 ELISA.

Results: Transfection of 16HBE14O$^-$ cells with 30nM IL-8 siRNA for 48 hours led to an 84% knockdown in IL-8 mRNA ($p<0.05$). Treatment with LPS (10µg/ml, 6h) increased IL-8 gene expression 1.5-fold ($p<0.05$) and IL-8 siRNA caused a significant knockdown of IL-8 protein in LPS-treated cells ($p<0.05$). Furthermore, basal IL-8 protein production was decreased by IL-8 siRNA. LPS treatment increased IL-8 protein expression in 16HBE14O$^-$ cells. IL-8 siRNA failed to decrease this effect.

Conclusions: The data show that IL-8 siRNA can inhibit basal and LPS-induced IL-8 gene expression in 16HBE14O$^-$ cells. Further investigation is required to optimise the conditions for inhibition of IL-8 protein production, and to study its potential role in CF therapy.

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Cystic fibrosis (CF) is a genetic disease characterised by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR). It is a neutrophil-dominated disease characterised by inflammation and mucus overproduction in the lungs. Furthermore, CF patients are prone to infection, particularly with *Pseudomonas aeruginosa*, which is an independent risk factor for mortality in CF patients.[1] The neutrophil chemokine interleukin-8 (IL-8) is present at higher than normal levels in the CF lung and is a key factor in neutrophilic infiltration of the lung (**Figure 1**). Neutrophil elastase (NE) is partially responsible for high IL-8 levels, as it overwhelms the normal anti-protease defence of the respiratory epithelial surface and leads to the upregulation of pro-inflammatory chemokines, including IL-8.[2] Neutrophil elastase increases the secretion of IL-8 from epithelial cells through interaction with Toll-like receptors (TLRs) and leukotriene B4 (LTB4) released by macrophages that create a gradient of neutrophil chemoattraction.[3] As a result, there is overwhelming inflammation of the lung, and neutrophils cannot effectively clear the infection. Through this molecular mechanism, NE and IL-8 play a significant role in the pathophysiology associated with CF.[3]

Many therapies that target a wide range of factors responsible for CF pathology have been investigated.[1-3] Wewers *et al.* investigated the feasibility and efficacy of alpha-1 antitrypsin (A1AT) replacement in A1AT-deficient emphysema patients and concluded that treatment was successful in the short term.[5] One important finding in this study was that A1AT inhibited NE, and it was this finding that initiated the investigation of the benefits of A1AT supplementation in CF therapy.[5] Further investigation showed that A1AT supplementation can also reduce IL-8 levels, presenting a potential therapy for CF patients.[6]

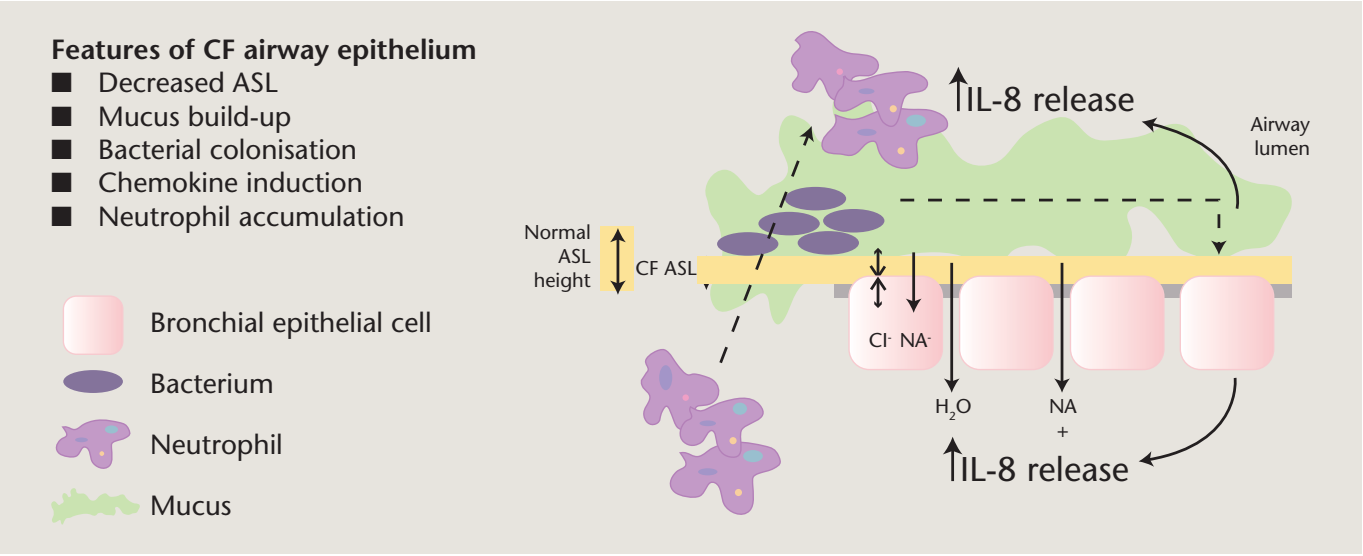

FIGURE 1: Features of CF airway epithelium. Defective CFTR function leads to decreased and dehydrated airway surface liquid (ASL) on the airway epithelium. Increased mucus production facilitates microbial colonisation with Pseudomonas *and other species. Pathogen-associated molecular patterns activate Toll-like receptor (TLR) signalling in bronchial epithelial cells to increase expression of the neutrophil chemokine IL-8, causing neutrophils to be recruited into the lumen of the lung. These neutrophils can secrete factors such as neutrophil elastase that further activate the airway epithelium, leading to a vicious cycle of inflammation.*[4]

Studies have since proven that A1AT replacement therapy (augmentation therapy) causes the inhibition of proteases and improves bactericidal processes in CF patients.[2]

Naturally occurring anti-proteases and NE inhibitors, such as secretory leucoprotease inhibitor (SLPI), Elafin, monocyte neutrophil elastase inhibitor (MNEI) and eglin-c, also represent potential therapies that may suppress IL-8 expression in CF lungs.[3] However, the effect of inhibiting the expression of IL-8 in human bronchial epithelial cells using small-interfering RNA (siRNA) remains unknown. siRNA can reduce gene expression through RNA interference (RNAi), an enzyme-mediated process that involves RNA-protein interactions characterised by four major steps: assembly of siRNA with RNA-induced silencing complex; activation of the complex; target recognition; and, target cleavage.[7] The manipulation of IL-8 expression using siRNA could represent a new gene therapy approach for CF.

The aims of this project are: (1) to grow and transfect 16HBE14O$^-$ cells with siRNA lengths designed to knockdown IL-8 gene expression; (2) to quantify the IL-8 expression; and, (3) to compare the extent of IL-8 knockdown in normal and LPS-stimulated bronchial epithelial cells. The effect of siRNA on IL-8 expression will be tested in time-course, dose response and combination experiments.

Methods

Cell culture

Cell line

16HBE14O$^-$ cells were maintained at 37°C in a 5% CO_2 incubator and supplemented with MEM-GlutaMax (Gibco, 1x) mixed with 10% foetal calf serum (FCS) and 1% penicillin-streptomycin (P/S).

Splitting HBE cells

Cells were grown in a 25cm^2 Corning flask pre-coated with 3mL fibronectin until 50-80% confluent. They were split using Dulbecco's phosphate buffered saline (DPBS, Gibco, 1x) and polyvinylpyrrolidone-EGTA-trypsin (PET) solution. The PET solution contained Hepes buffered saline solution (HBSS), 10% polyvinylpyrrolidone solution (PVP), 0.2% EGTA in HBSS and 0.25% trypsin in 0.02% EDTA. Cells were counted using Glasstic (KOVA) haemocytometers and placed onto a cell culture cluster plate (Costar) at a concentration of 1.5 x 10^5 cells/well.

Cell treatment

siRNA transfection

Each well containing 16HBE14O$^-$ cells was treated with 250µl of MEM-GlutaMax +FCS +P/S and 50µl of siRNA transfection reagents. The siRNA reagents consisted of Opti-MEM (Gibco) mixed in solution with RiboJuice (Novagen) and the siRNA of interest at a concentration of 30nm/well. Either silencer negative control siRNA, silencer GAPDH siRNA or silencer IL-8 select validated siRNA (Ambion) was used. Each siRNA transfection was carried out in triplicate. Once transfected, the cells were incubated for 24-48 hours in an incubator at 37°C in 5% CO_2. In some experiments, supernatants were removed and stored at -4°C after incubation was complete.

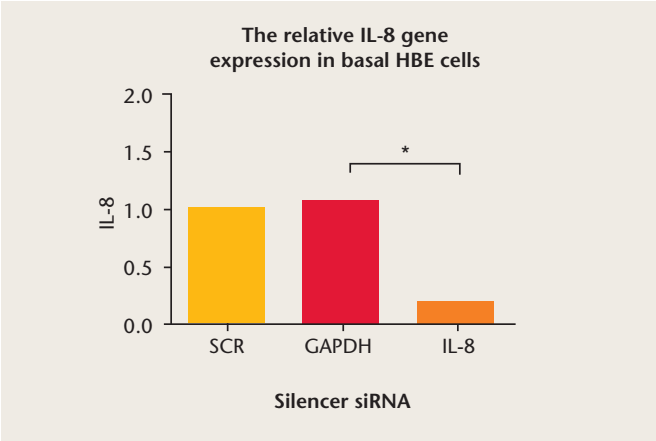

*FIGURE 2a: Effect of various siRNA on quantitative IL-8 gene expression in basal 16HBE14O- cells relative to β-actin. 16HBE14O- cells (2x10^5/well) were transfected with the siRNA of interest (in triplicate) and incubated for 48 hours. Relative expression of IL-8 was quantified by qRT-PCR using β-actin (SCR) as a reference gene (*p<0.05).*

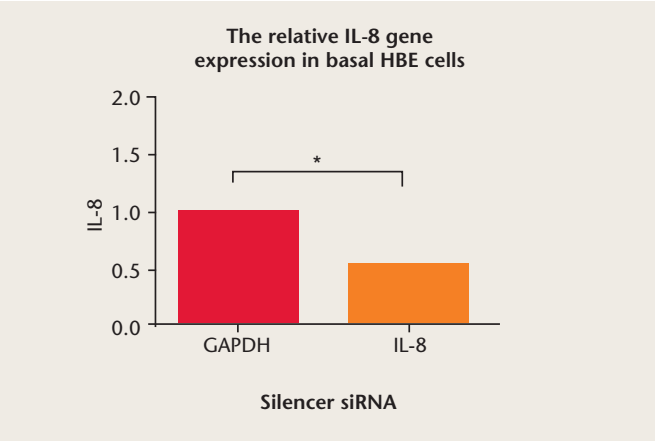

*FIGURE 2b: Effect of GAPDH and IL-8 siRNA on quantitative IL-8 gene expression in basal 16HBE14O- cells relative to GAPDH. 16HBE14O- cells were transfected with the siRNA of interest (in triplicate) and incubated for 48 hours at an optimal cell density of 1.5x10^{5}cells/well. Relative expression of IL-8 was quantified by qRT-PCR using GAPDH as a reference gene (*p<0.05).*

LPS treatment

Cells were further stimulated with LPS from *Pseudomonas aeruginosa* (Sigma-Aldrich). Cells were seeded into 18 wells of a cell cluster plate (Costar) and transfected with the siRNA of interest. Cells were incubated at 37°C in 5% CO_2 for 24 hours, following which supernatants were removed and stored at -20°C. Of the 18 wells, nine were supplemented with 1mL 1% FCS-MEM-GlutaMax. The other nine were treated with 1mL LPS (10µg/mL) in solution with 1% FCS-MEM-GlutaMax. The cells were incubated for six hours at 37°C in 5% CO_2. After incubation, supernatants were removed and stored at -20°C.

RNA isolation

RNA was isolated from the 16HBE14O- cells using 500µl/well TRI reagent (Sigma-Aldrich) as per the manufacturer's protocol. This was followed by phase separation using chloroform (100µl/sample, Sigma-Aldrich), RNA isolation using isopropanol (250µl/sample, Sigma), washing of the RNA pellet in 75% ethanol and dissolution in 0.1% diethyl pyrocarbonate (Sigma-Aldrich) H_2O as per the manufacturer's protocol.

cDNA synthesis and qRT-PCR

RNA quantification was carried out using a Nanodrop and equal quantities of RNA were reverse transcribed into cDNA using the Quantitect Reverse Transcription kit (Qiagen). The resulting cDNA was used for quantitative real-time (qRT) PCR.

A 1x qRT-PCR mastermix was made up to 20µl and added to each well of the 96-well PCR plate. The 1x mix consisted of 2µl template cDNA, 10µl of SyBr Green (Roche), 6µl of PCR H_2O (Roche) and 1µl each of a forward and reverse primer (10pmol/µl β-actin, GAPDH or IL-8; Roche). Amplification was performed using the LightCycler 480 PCR system (Roche) at an annealing temperature of 56°C for 45 cycles. Quantitative expression of the target gene was determined using the $2^{(-\Delta\Delta Ct)}$ method.[8,9] Statistical analysis was conducted using two different algorithms – one generating a single value from triplicate data and the other comparing individual values to a single standard to generate triplicate data.

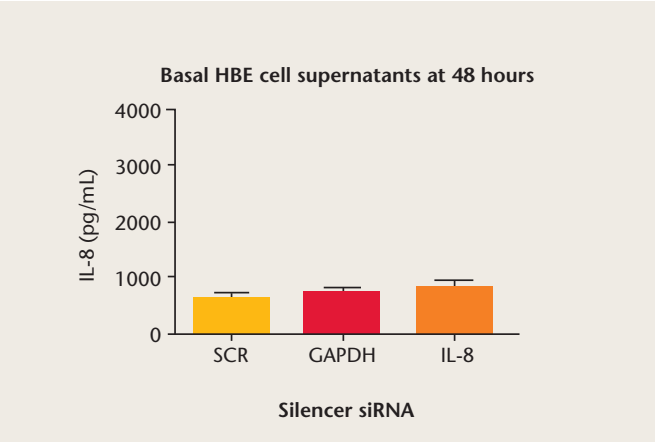

FIGURE 3: IL-8 protein production in supernatants of basal 16HBE14O- cells. Supernatants were acquired from 16HBE14O- cells 48 hours after siRNA transfection. Amount of IL-8 protein was measured using ELISA at an absorbance of 405nM for 0.1 seconds (p=0.4).

Enzyme-linked immunosorbent assay (ELISA)

The supernatants acquired from the cell culture plates after siRNA transfection/LPS treatment were measured for the quantity of IL-8 protein (pg/mL) produced using the Sandwich ELISA protocol.[10] Spectrophotometry was used to measure IL-8 at an absorbance of 405nm at 0.1 seconds.

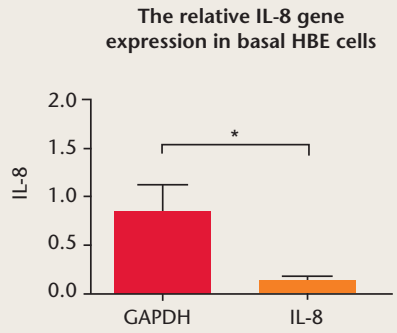

*FIGURE 4a: Effect of GAPDH and IL-8 siRNA on quantitative IL-8 gene expression in basal 16HBE14O- cells relative to GAPDH. 16HBE14O- cells (1.5x10^5/well) were transfected with the siRNA of interest (in triplicate) and incubated for 30 hours. Relative expression of IL-8 was quantified by qRT-PCR using GAPDH as a reference gene (*p<0.05).*

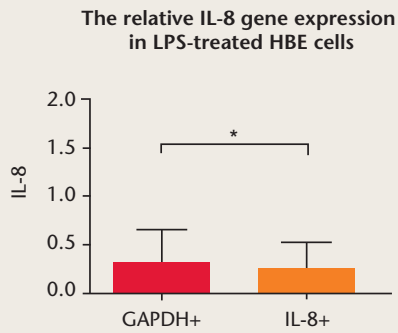

FIGURE 4b: Effect of GAPDH and IL-8 siRNA on quantitative IL-8 gene expression in LPS treated 16HBE14O- cells relative to GAPDH. 16HBE14O- cells (1.5x10^5/well) were transfected with the siRNA of interest (in triplicate) and incubated for 24 hours. They were then treated with LPS and incubated for another six hours. Relative expression of IL-8 was quantified by qRT-PCR using GAPDH as a reference gene (p=1.0).

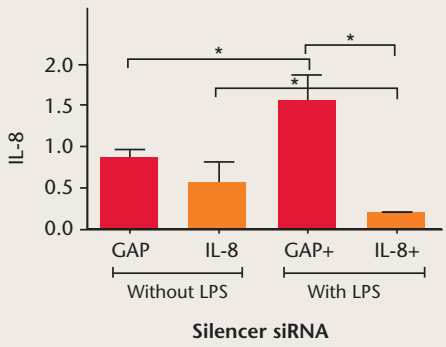

*FIGURE 5: Comparison between the relative gene expressions of IL-8 in basal 16HBE14O- cells and LPS treated 16HBE14O- cells (*p<0.05). In untreated cells, no IL-8 siRNA-mediated reduction in IL-8 expression was detected (p=1.0)*

Statistical analysis

Assays were performed in triplicate and the data was analysed by Prism 4.0 using student t-test.

Results

The objective of these experiments was to observe a knockdown of IL-8 gene expression in 16HBE14O- cells using siRNA. The quantitative gene expression of IL-8 and the production of IL-8 protein were measured by RT-PCR and ELISA, respectively. The effects of siRNA on IL-8 gene and protein expression were investigated in both basal and LPS-stimulated 16HBE14O- cells.

Knockdown of IL-8 in basal HBE cells

Duplicate experiments were carried out to measure the knockdown of basal IL-8 expression in 16HBE14O- cells that were transfected in triplicate with a negative control scrambled β-actin siRNA (SCR), GAPDH siRNA or IL-8 siRNA. **Figure 2a** shows a significant 80% knockdown in IL-8 (0.20) with β-actin as the control (p<0.05). Because of the similarity in expression of the β-actin (1.00) and GAPDH (1.06) reference genes (**Figure 2a**), GAPDH was subsequently chosen as the control to compare the relative knockdown of IL-8 (**Figure 2b**). **Figure 2b** demonstrates a 50% IL-8 siRNA-mediated knockdown in IL-8 (p<0.05). Furthermore, the optimal cell density of 16HBE14O- cells was found to be 1.5x10^5 cells/well (data not shown); this cell density was used in further experiments.

IL-8 protein production in basal HBE cells

After the 16HBE14O- cells were transfected with siRNA and incubated for 48 hours, the supernatants were isolated and IL-8 protein production was assessed using ELISA (**Figure 3**). IL-8 protein was increased (824pg/mL (95% CI 725-923)) in the supernatants of 16HBE14O- cells transfected with IL-8 siRNA compared to cells transfected with SCR siRNA (618pg/mL (95% CI 544-690)), but not to those transfected with GAPDH siRNA (698pg/mL (95% CI 614-782)).

Knockdown of IL-8 in HBE cells treated with LPS

Further experiments were carried out to compare the knockdown of IL-8 in untreated 16HBE14O- and LPS-treated 16HBE14O- cells (**Figure 4**). An 84% knockdown in IL-8 in basal 16HBE14O- cells was documented (p<0.05), as seen previously. No statistically significant knockdown in IL-8 was witnessed in 16HBE14O- cells treated with LPS (p=1.0).

A duplicate experiment was carried out (**Figure 5**) comparing the effect of IL-8 siRNA against the GAPDH control siRNA on knockdown

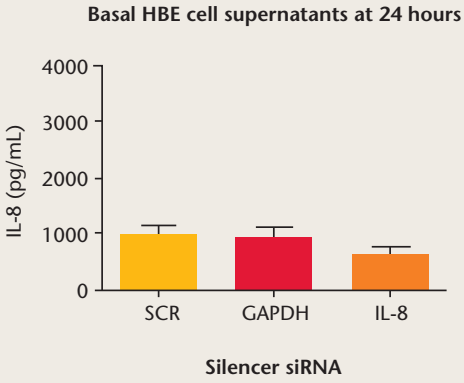

FIGURE 6a: IL-8 protein production in supernatants of basal 16HBE14O- cells at 24 hours post siRNA transfection. Supernatants were acquired from 16HBE14O- cells 24 hours post siRNA transfection. Amount of IL-8 was measured using ELISA at an absorbance of 405nM for 0.1 seconds (p=0.14).

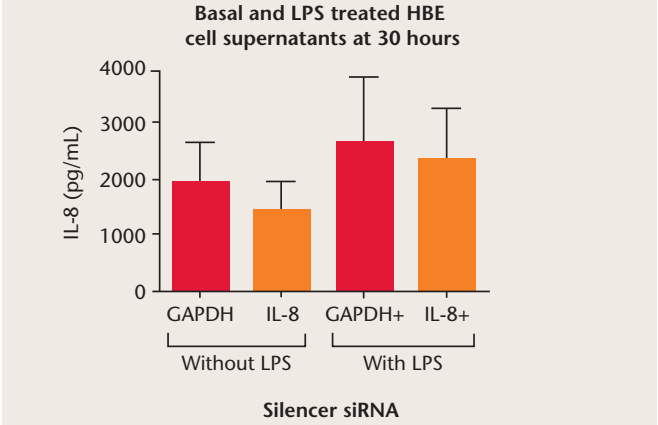

FIGURE 6b: IL-8 protein production in supernatants of basal 16HBE14O- cells and treated 16HBE14O- cells at 30 hours post siRNA transfection. Supernatants were acquired from both basal and LPS-treated 16HBE14O- cells 30 hours post siRNA transfection. Amount of IL-8 protein was measured using ELISA at an absorbance of 405nM for 0.1 seconds (without LPS, p=0.5; with LPS, p=0.9).

of IL-8 gene expression. IL-8 siRNA did not significantly knock down basal IL-8 expression (30% reduction; p=1.0). There was a significant increase in the expression of IL-8 in LPS-treated cells (1.55) as compared to basal cells (0.90) transfected with GAPDH siRNA (p<0.05). There was also a significant knockdown of IL-8 in LPS-treated cells (p<0.05).

IL-8 protein production in LPS-treated HBE cells

The supernatants acquired at 24 hours and 30 hours post siRNA transfection were measured for IL-8 knockdown using ELISA. IL-8 protein expression in 16HBE14O- cells transfected with IL-8 siRNA is not significantly knocked down in comparison to cells transfected with SCR and cells transfected with GAPDH siRNA (**Figure 6a**) (p=0.14). In **Figure 6b**, untreated 16HBE14O- cells are compared to LPS-treated 16HBE14O- cells. There is an increase in the amount of IL-8 protein produced in LPS-treated cells compared to basal cells for cells transfected with GAPDH siRNA and cells transfected with IL-8 siRNA, yet the knockdown in IL-8 remains evident. Although it appears as though there is a knockdown of IL-8 protein production, there was no significant difference between the GAPDH or IL-8 siRNA-transfected cells (p=0.4) (**Figure 6c**).

Discussion

The inflammatory chemokine IL-8 is a key factor responsible for the infiltration of neutrophils in the CF lung. In this project, the efficacy of siRNA in knocking down basal and LPS-induced IL-8 gene and protein expression in human bronchial epithelial cells was explored. A knockdown in basal IL-8 gene expression between 50% and 84% was seen in basal HBE cells transfected with IL-8 siRNA (**Figures 2a, 2b** and **4a**). These results were reproducible and statistically significant (p<0.05). This indicates that IL-8 siRNA is very effective in inhibiting IL-8 gene expression in normal human bronchial epithelial cells.

Furthermore, a statistically significant knockdown in IL-8 gene expression was seen in LPS-induced HBE cells transfected with IL-8 siRNA (p<0.05) (**Figure 5**). These positive results reiterate the efficacy of siRNA, and put it forth for gene therapy for CF patients in the future.

The results of IL-8 inhibition are consistent with findings demonstrated in a study conducted by Tsivkovskii *et al.* in which the effect of a quinolone on IL-8 expression was investigated.[11] The effect of levofloxacin on pro-inflammatory cytokine secretion by human airway epithelial cells *in vitro* was examined, and results demonstrated a dose-dependent reduction in IL-8 in both tumour necrosis factor α (TNFα) and LPS-induced human airway epithelial cells. This suggests that levofloxacin could also be of therapeutic benefit in CF. Further studies demonstrated that IL-8 suppression can be successfully accomplished by several techniques.[12,13]

An extensive knockdown of IL-8 gene expression from 1.55 (95% CI 1.3-1.8) to 0.19 (95% CI 0.19-0.20) (p<0.05) was seen in LPS-induced cells (**Figure 5**). The magnitude of knockdown in LPS-induced cells exceeded that witnessed in basal cells; this result was unexpected, as LPS has been documented to stimulate the production of IL-8.[14] It must be noted that these results were not reproduced. Further experiments should be carried out under the same conditions to attain a truer representative of the IL-8 knockdown value in LPS-induced HBE cells.

The amount of basal IL-8 protein production detected using ELISA did not differ in a statistically significant manner (**Figures 3** and **6a**). However, the trends in the duplicate experiments conflicted; repeat experiments are needed to demonstrate reproducibility.

Although there appeared to be a small decrease in LPS-induced IL-8 protein production by the IL-8 siRNA (**Figure 6b**), on further investigation, by measuring the change in IL-8 (**Figure 6c**), it was found that there was in fact no decrease. This could be due to error

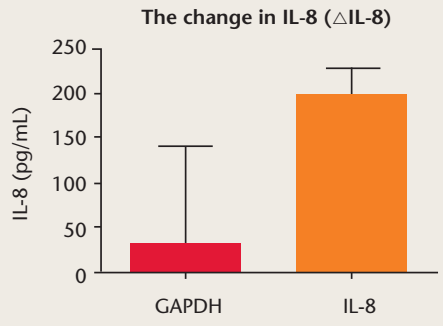

*FIGURE 6c: The change in IL-8 (△IL-8) protein production between supernatants of basal 16HBE14O- cells and treated 16HBE14O- cells. The change in IL-8 protein production between basal 16HBE14O- cells and LPS-treated 16HBE14O- cells (as measured in **Figure 3b**) was calculated (p=0.4).*

in technique; although the sandwich ELISA is quite sensitive, it involves indirect antibody detection and there is a risk of cross-reactivity resulting in a non-specific signal.[10] As such, it is unclear whether the IL-8 siRNA has a significant effect on basal or LPS-induced IL-8 protein production.

IL-8 protein expression patterns detected in the study differ from previous findings. Von Scheele *et al.* tested the efficacy of budesonide in inhibiting IL-8 in bronchial epithelial cells treated with organic dust, TNF or LPS using ELISA.[15] Results showed that budesonide suppressed the secretion of IL-8 in cells treated with all three stimuli.

Conclusions and future recommendations

A successful knockdown in basal and LPS-induced IL-8 gene expression was seen in HBE cells. The effect of IL-8 siRNA on basal and LPS-induced IL-8 protein production remains unclear and should be further investigated using varying doses of siRNA and longer transfection times. Future work should aim to explore the efficacy of IL-8 siRNA in different types of cells and growth environments. The next steps could be investigating the knockdown of IL-8 using siRNA in an air-liquid interface culture or the use of animal models.[16] Furthermore, the effect of siRNA in CF airway epithelial cells and human monocytes should be explored to gauge the suitability of siRNA as a therapeutic modality in the CF patient.

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