Down-regulation of IL-8 expression in human airway epithelial cells through helper-dependent adenoviral-mediated RNA interference

Huibi CAO1,4, Anan WANG1, Bernard MARTIN1, David R. KOEHLER1,4, Pamela L. ZEITLIN1, A. Keith TANAWELL1,2,3, Jim HU1,2,4,*

1Programme in Lung Biology Research and the Canadian Institutes of Health Research Group in Lung Development, Hospital for Sick Children, Toronto, Canada M5G 1X8.
2Department of Paediatrics, University of Toronto, Toronto, Canada M5S 1A1
3Department of Physiology, University of Toronto, Toronto, Canada M5S 1A1
4Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada M5S 1A1
5Departments of Pediatrics, The Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA.

ABSTRACT

Interleukin (IL)-8 is a potent neutrophil chemotactic factor and a crucial mediator in neutrophil-dependent inflammation. Various cell types produce IL-8, either in response to external stimuli such as cytokines or bacterial infection, or after malignant transformation. Anti-IL-8 strategies have been considered for anti-inflammatory therapy. In this paper we demonstrate that the RNA interference technique can be used to efficiently down-regulate IL-8 protein expression in airway epithelial cells. We used a helper-dependent adenoviral vector to express a small hairpin (sh)RNA targeting human IL-8 in cultured airway epithelial cells (IB3-1, Cftr-/-; C38, Cftr-corrected) stimulated with TNF-α, IL-1β or heat-inactivated Burkholderia cenocepacia. Stimulated IL-8 expression in IB3-1 and C38 cells was significantly reduced by shRNA expression. The shRNA targeting IL-8 had no effect on the activation of NF-κB, or on the protein levels of IκB or IL-6, suggesting that this anti-IL-8 strategy was highly specific, and therefore may offer potential for the treatment of inflammatory diseases.

Keywords: Interleukin-8, RNA interference, helper-dependent adenoviral vector, inflammation, chemokine, neutrophil, cystic fibrosis.

INTRODUCTION

IL-8 is a potent neutrophil chemoattractant and activator [1], and high levels of IL-8 are frequently found to be associated with a variety of inflammatory diseases with neutrophil infiltration. For example, in airways of cystic fibrosis patients, the production of IL-8 is exaggerated, leading to excessive neutrophil infiltration [2]. Patients with ulcerative colitis display increased levels of IL-8 in inflamed areas of the gut [3]. In addition, IL-8 is induced in synovial cells of rheumatoid arthritis patients [4], and in the myocardium after ischemia and reperfusion in vivo [5]. Interestingly, elevated levels of IL-8 in the sputum of cystic fibrosis patients is reduced by either antibiotic therapy or gene therapy with AAVCFTR [6, 7]. In certain gene therapy applications inflammation due to the therapy is a problem [8], and addition of an anti-IL-8 treatment may be helpful.

IL-8, also called CXCL8, belongs to the CXC chemokine family [9]. IL-8 expression is tightly regulated and requires activation of NF-κB as well as MAP kinases [10, 11]. Certain cytokines, such as TNF-α and IL-1β, and many microbial products including lipopolysaccharide, tri-acyl lipopeptides and bacterial flagellin, are potent inducers of IL-8 expression in a variety of cells, such as macrophages and epithelial cells. Induction of IL-8 by TNF-α or IL-1β is mediated through their corresponding receptors, which activate the NF-κB signaling pathway [12, 13]. Similarly, microbial products induce IL-8 expression via a family of plasma membrane receptors called Toll-like receptors.
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**Materials and methods**

**Cell culture**

IB3-1 (AF508/w1282X) and C38 cells (CFTR-corrected) were described in [31, 32]. IB3-1 and C38 cells were cultured in LHC-8 medium containing 5% FBS and antibiotics (2.5 µg/ml fungizone, 80 µg/ml tobramycin, 0.2mg/ml primaxin and 100U/ml each of penicillin and streptomycin). COS-1 cells were grown in DMEM with 10% FBS.

**IL-8 RNAi design**

An siRNA-expressing construct was designed as described [25], using a mouse U6 gene promoter to drive expression. A target region of IL-8 was selected beginning with three guanines (5'-GGG TGC AGA GGG TTG TGG AGA-3'). Shown in Fig. 1A, top, two sets of DNA oligonucleotides were used to construct the coding region for the short double-stranded RNA with a hairpin. A HindIII site was designed at the hairpin to facilitate restriction analysis for the cloning. In the second pair of oligonucleotides, the sequence of the top strand ends with TTTTT, which serves as Pol III transcription termination signal. The plasmid pBSII-SK (Stratagene, La Jolla, CA) was used as the backbone for the final plasmid, named pBS/U6-IL8. All DNA oligonucleotides were purchased from ACTG Inc. (Toronto, ON, Canada).

**Reporter system for measuring gene knock-down**

A reporter plasmid for measuring IL-8 knock-down was constructed by inserting a 21 bp double-stranded oligonucleotide containing the IL-8 targeted site into the pCMV-SEAP reporter plasmid, between the CMV promoter and the translation initiation codon of the secreted alkaline phosphatase (SEAP) gene in pSEAP2 (Clontech, Palo Alto, CA) (Fig. 1A, bottom). The resulting plasmid, pCMV-IL8SEAP, expresses the same level of SEAP activity as its parental plasmid, pCMVSEAP. COS-1 cells were cotransfected with pCMV-IL-8SEAP and pBS/U6-IL8RNAi in a ratio of 1:30 (by moles) using PolyFect Transfection Reagent (QIAGEN, Toronto, Canada). Cell culture media were collected for SEAP analysis 48 h following cotransfection, and SEAP assays were performed using the Phospho-Light System (Applied Biosystems, Bedford, MA).

**HD-Ad-IL8RNAi production**

A BssHII-DNA fragment containing the U6 promoter and a short sequence for IL-8 RNAi expression was excised from pBSII-U6/IL-8RNAi and ligated into the AscI site of pC4HSU (Merck and Co., USA) (Supplementary Fig. 1). The IL-8RNAi-C4HSU DNA was treated with Pmel to expose the viral ITRs and introduced into 293t cells using the Cellfect Transfection kit (Amersham Pharmacia, Buckinghamshire, UK). The preparation and purification of viral vectors were carried out as described [33].

**Viral transduction**

To determine the number of virus particles per cell required to achieve transduction of ~100% cells, we tested transduction frequency using 0, 25, 50 and 100 moi (number of infectious units per cell) of a lac-Z-expressing HD-Ad. We found that 50 moi can achieve almost 100% transduction of IB3-1 and C38 cells.

**Induction and ELISA**

IB3-1 and C38 cells were treated with (or without) HD-AD-IL8RNAi for 4 h, then induced for 24 h with 5 ng/ml TNF-α, or...
1 ng/ml IL-1β, or 50 cfu of *Burkholderia cenocepacia* (Bc; clinical isolate BC7, strain ET12) that was heat inactivated (20 min at 60°C). Human recombinant TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN). Conditioned media were collected and stored at -80°C. IL-8 and IL-6 protein levels were measured by ELISA (Bender MedSystems, Diagnostics GmbH, Vienna). The statistical analysis was performed with the two-tailed t-test.

**EMSA**

Nuclear extracts were prepared as described [34]. A DNA oligonucleotide probe containing the κ-light chain enhancer NF-kB consensus binding site (5'-AGTTGAGGGGACTTTCCCAGG C-3') was end-labelled with γ-32P-ATP. Five micrograms of nuclear extracts were used for each reaction. Complexes were resolved on 6% non-denaturing polyacrylamide gels.

**RESULTS**

Analysis of shRNA targeting efficiency using a reporter gene system

We built an shRNA expression plasmid, pBS/U6-IL-8RNAi, using an U6 promoter-based plasmid vector [25] to produce an shRNA targeting the human IL-8 mRNA (Fig. 1A, top). To determine the silencing efficiency of the shRNA, we designed a SEAP reporter plasmid with an IL-8 target sequence at the 5’ end of the SEAP coding region of pCMV-SEAP (Fig. 1A, lower). The level of SEAP expression from the target plasmid, pCMV-IL-8-SEAP,
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Fig. 2 Reduction of cytokine-induced IL-8 expression by shRNA expressed from a helper-dependent vector. IB3-1 cells (A) or C38 cells (B) were infected with IL-8RNAi-HD-Ad or HD-Ad-LacZ (as a viral vector control) and induced with 5 ng/ml TNF-α or 1 ng/ml IL-1β. IL-8 protein was analyzed by ELISA. The average of three independent experiments is shown; error bars indicate mean ± SE. *P < 0.05, TNF-α or IL-1β induced cells receiving IL-8RNAi-HD-Ad versus induced cells not receiving HD-Ad.

was comparable to that from the parental plasmid when co-transfected with the control plasmid pBS/U6 into COS-1 cells (Fig. 1B). To determine whether pBS/U6-IL8RNAi was functional, we introduced it together with the reporter target plasmid (pCMV-IL-8-SEAP) into COS-1 cells. pBS/U6-IL8RNAi significantly reduced SEAP production from pCMV-IL-8-SEAP (~80%). pBS/U6-IL8RNAi had no effect on SEAP expression from the parental plasmid (pCMV-SEAP) which does not contain the IL-8 target sequence. As reported in other studies [23, 25], our co-transfection analysis was done with a high ratio of RNAi/target plasmid (30:1). To enhance the transduction efficiency and stability we decided to use an HD-Ad to deliver the IL-8 RNAi cassette.

IL8RNAi-HD-Ad reduces endogenous IL-8 expression induced by TNF-α or IL-1β

The reporter analysis indicated indirectly that the shRNA expressed from pBS/U6-IL8RNAi can knock down IL-8 mRNA. We next designed an HD-Ad vector (Supplementary

Fig. 3 Reduction of bacterium-induced IL-8 expression by shRNA expressed from a helper-dependent vector. IB3-1 or C38 cells transduced with IL-8RNAi-HD-Ad were stimulated with heat-inactivated B. cenocepacia (Bc) for 24 h. IL-8 protein was determined by ELISA. The average of three independent experiments is shown; error bars indicate mean ± SE. *P < 0.05, cells receiving IL-8RNAi-HD-Ad versus those not receiving HD-Ad.

IL8RNAi-HD-Ad reduces endogenous IL-8 expression induced by heat-inactivated bacteria

Burkholderia cenocepacia (bacteria belonging to genomovar III of the Burkholderia cepacia complex) is an opportunistic pathogen found in patients with cystic fibrosis and induces IL-8 production in lung epithelial cells [11]. We examined whether IL8RNAi-HD-Ad could knock down B. cenocepacia-induced IL-8 expression in airway epithelial cells. We heat-inactivated the bacteria so their numbers could be easily controlled in cell culture. Shown in Fig. 3, the level of IL-8 expression in either cell line (data not shown). The control virus HD-Ad-lacZ did not induce expression of IL-8 (Fig. 2A, B), nor did it affect TNF-α or IL-1β induction of IL-8 in both cell lines was very low (undetectable in C38) under normal growth conditions, but was induced in the presence of TNF-α or IL-1β. Shown in Fig. 2A and B, the level of IL-8 protein was 3-4.5-fold higher in CF cells (IB3-1) than in Cfr-corrected cells (C38) when induced by TNF-α or IL-1β. IL-8 induction by TNF-α or IL-1β in each cell line was inhibited ~70% when the cells were pre-treated with IL8RNAi-HD-Ad. Similar results were obtained following cytokine stimulation for 48 hours (data not shown).
IL8RNAi-HD-Ad-mediated IL-8 reduction does not affect NF-κB activation.

Previous studies have demonstrated that TNF-α or IL-1β stimulate expression of IL-8 and IL-6 through activation of the NF-κB pathway [12, 35]. Although the RNAi strategy targeting IL-8 is expected to be gene-specific, non-specific down-regulation of NF-κB activity could also reduce the level of IL-8 expression. To test this possibility, we performed EMSA to examine the NF-κB DNA-binding activity in nuclear extracts from cells receiving various treatments. IL8RNAi-HD-Ad did not interfere with TNF-α or IL-1β induced NF-κB activation in IB3-1 or C38 cells (Fig. 4A, lanes 5, 7 and lanes 11, 13). There was no change in NF-κB DNA-binding activity in un-induced cells, regardless of whether they were first transduced with IL8RNAi-HD-Ad (Fig. 4A, lanes 8 and 14). Similarly, IL8RNAi-HD-Ad did not interfere with Bc-induced NF-κB activation (Fig. 4B). Under quiescent conditions NF-κB is inhibited by binding to IκB in the cytoplasm. Upon stimulation IκB is degraded, allowing NF-κB to translocate to the nucleus and modulate inflammatory gene expression [36]. Both IκB-α and IκB-β were degraded quickly (in 15 min) following stimulation with TNF-α or IL-1β, regardless of whether the cells were first transduced with IL8RNAi-HD-Ad. IL8RNAi-HD-Ad had no effect on the levels of IκB-α and -β in uninduced cells (Fig. 5).

IL8RNAi-HD-Ad does not affect IL-6 induction

Expression of both IL-6 and IL-8 is induced by TNF-α or IL-1β through NF-κB activation [12]. To examine
whether IL-6 induction by TNF-α or IL-1β is non-specifically affected by IL-8RNAi, we measured IL-6 activity in IB3-1 and C38 cells transduced with IL-8RNAi-HD-Ad. Shown in Fig. 6, there was a very low level of IL-6 protein secreted from the unstimulated cells. IL-6 protein was greatly induced by incubation with TNF-α, IL-1β or Bc for 24 h. However, there was no difference in expression of IL-6 between cells treated with or without IL-8RNAi-HD-Ad. In contrast to IL-8, there was little difference in IL-6 induction between CF and Cfr-corrected cells.

**DISCUSSION**

Induction of IL-8 occurs in a variety of inflammatory diseases, including cystic fibrosis, chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), asthma, idiopathic pulmonary fibrosis, meconium aspiration syndrome, bacterial pneumonia, psoriasis, rheumatoid arthritis, inflammatory bowel disease and septic shock [10, 37-41]. Boekholdt et al. demonstrated that among apparently healthy men and women, elevated levels of IL-8 are associated with an increased risk of future coronary artery disease [42]. Recent reports also indicate that IL-8 plays an important role in atherosclerosis, and in mediating angiogenic activity in tumor growth and metastasis [19, 43, 44].

Strategies for inhibiting IL-8 include neutralizing the protein with fully humanized antibodies against IL-8 [45, 46]. In a rabbit model of lung reperfusion injury, reperfusion of the ischaemic lung induced local IL-8 production, massive neutrophil infiltration and destruction of pulmonary tissue. Administration of a neutralizing monoclonal antibody against IL-8 prevented neutrophil infiltration and tissue injury [18]. IL-8 antibody administration also significantly inhibited proteinuria, as well as infiltration of neutrophils into rabbit glomeruli, in an acute glomerulonephritis animal model initiated by deposition of immune complex [20]. In a mouse orthotopic bladder cancer model, a significant decrease in human tumor growth and invasion was observed after IL-8 antibody treatment [19, 47]. These observations suggest that IL-8 could be a target for therapeutic intervention in neutrophil-mediated acute inflammation, and possibly carcinogenesis. But the use of humanized antibodies in clinical applications is hindered by a combination of factors including a brief half-life and fast blood clearance, their immunogenicity, and high cost.

Recent advances in sequence-specific gene silencing by double-stranded RNAs (RNA interference) provides a new platform to develop gene-specific drugs for disease management. Our results indicated that an IL-8RNAi expression plasmid significantly reduced the activity of a reporter gene (SEAP) containing an IL-8 target sequence. Although the use of plasmid-based shRNA expression has quickly become the preferred method for targeted gene silencing, there are limitations. The delivery of plasmids to certain types of cells such as primary cells, neural cells and stem cells is hampered by poor transfection efficiency. Delivery of expression plasmids to mammals as gene therapy is very inefficient, particularly to the lung [48].
The delivery of shRNA by viral transduction is one way to overcome these shortcomings. Some groups [49-52] have reported that efficient introduction and stable integration of shRNA-expression cassettes can be achieved using Moloney murine leukemia virus, lentiviral vector systems or the first-generation adenoviral vectors. However, the first-generation Ad vector is not a good choice for delivery of anti-inflammatory shRNAs to mammals, since the vector itself provokes strong host inflammatory responses. HD-Ads cause significantly less inflammation in mammals since they do not express adenoviral genes, although some inflammation due to the capsid proteins still occurs. Like earlier generations of Ads, HD-Ads transduce most cell types very effectively. In addition, the integration frequency of vector DNA into the host genome is very low [53], thus reducing risk of insertional mutagenesis in clinical applications. One concern about using HD-Ads, versus first-generation Ads, is the technical difficulty in producing these vectors. However, Palmer and Ng reported a new method that can shorten the production time of HD-Ads to 12 days, and produce >10^13 viral particles [54].

Microbial products can activate the NF-κB signaling pathway in airway epithelial cells [55, 56]. This activation is initiated through interactions between microbial products and various Toll-like receptors present in airway epithelial cells [57]. Activation of the NF-κB pathway via TLRs can induce the expression of cytokines, including IL-8, which leads to inflammation. Furthermore, since IL-8 is a potent chemoattractant and activator of neutrophils, overexpression of IL-8 can lead to excessive neutrophil infiltration and tissue damage. The level of IL-8 in CF airway is greatly enhanced compared to normal subjects [12, 13, 58, 59] and its overexpression may be a major cause of lung damage in CF patients. We demonstrated here that a clinically relevant Bc strain (heat-inactivated) can activate NF-κB in airway epithelial cells, leading to increased IL-8 production. Stimulation of the cells with TNF-α, IL-1β or bacteria induced a higher level of IL-8 expression in IB3-1 cells (Cftr−) than in C38 cells (Cftr+ corrected), in agreement with previous reports using these cell lines or primary cells [60, 61]. Although Cftr knock-out mice can be used as a model for Bc infection in CF [62-64], they are unfortunately not suitable for assessing our HD-Ad-IL8RNAi. There is no bona fide mouse homologue of human IL-8, and our IL-8 target sequence does not share sequence homology with rodent functional IL-8 homologues, such as MIP-2, KC or CINC-1. A large animal model may be useful in preclinical analysis of the HD-Ad-IL8RNAi.

Chi et al [65] demonstrated that shRNA-mediated gene silencing of mRNAs is highly gene-specific and does not produce secondary effects, as detectable by genome-wide expression profiling. However, it is possible that not all sequences are gene-specific, and therefore each shRNA should be experimentally examined for nonspecific effects. Although we cannot rule out all genome-wide effects from the experiments performed here, the HD-Ad-IL8RNAi did not appear to affect the NF-κB pathway, which regulates transcription of many cytokine genes including IL-8, nor did it affect IL-6 production, which is also regulated by the NF-κB pathway. Use of a control HD-Ad (expressing lacZ) revealed that neither IL-8 nor IL-6 were induced by the Ad particle itself in cultured cells, although this does not rule out the possibility of inducing inflammatory cytokines in other cell types, such as macrophages [66].

Since HD-Ads can effectively transduce cells in vivo and in vitro, a vector targeting the IL-8 mRNA could have important clinical implications. IL-8 is an essential component of the host defense system and it may not be a viable approach to block its activity systemically. Local administration of HD-Ad-IL8RNAi, for example to the lung, may be more practical. In addition, many delivery methods for gene therapy exacerbate inflammation. Our vector could be combined with other gene therapy vectors to minimize host inflammatory responses.

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