

IL-9 Modulated *MUC4* Gene and Glycoprotein Expression in Airway Epithelial Cells

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Abstract Compromised epithelial cell integrity is a common feature associated with chronic lung inflammatory states such as asthma. While epithelial cell damage is largely due to sustained effects of inflammatory mediators localized to airways, the subsequent process of epithelial cell differentiation is attributed to members of the transmembrane receptor tyrosine kinase family called the ErbB's. *MUC4*, a large molecular weight membrane-bound glycoprotein, has recently been identified as a potential ligand for the ErbB-2 receptor. In this study, we investigated the possible role of interleukin-9 (IL-9), a Th2 cytokine, on *MUC4* expression using a lung cancer cell line, NCI-H650. We determined that IL-9 up-regulates *MUC4* expression in a time and concentration-dependent fashion. Nuclear run-on assays indicated transcriptional regulation of *MUC4* while no post-transcriptional mRNA stabilization was observed by actinomycin D chase experiments. IL-9 also increased *MUC4* glycoprotein expression as determined by Western blots using a monoclonal antibody specific for a non-tandem repeat region on ASGP-2 region of *MUC4*. Furthermore, a JAK3-selective inhibitor 4-(4'-hydroxyphenyl) amino-6, 7-dimethoxyquinazoline (WHI-P131), substantially reduced IL-9-induced *MUC4* mRNA expression in a dose-dependent fashion. These results implicate a potential role for IL-9 upon *MUC4* expression in human airway epithelial cells.

Keywords *MUC4* · Mucins · IL-9 · Airway epithelium · ErbB2 · Asthma · Cystic fibrosis

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Introduction

Airway inflammation and compromised epithelial cell integrity are common phenomena associated with chronic airway diseases such as asthma. The airway epithelial lining of the human respiratory tract is composed of phenotypically distinct cell types, which play a vital role in regulating anti-protease defense and inflammatory responses [1]; however, persistent exposure of inflammatory cytokines, chemokines and proteolytic enzymes associated with an abnormal influx of immune cells in airway inflammatory disease states [2] affects the balance between epithelial cell death and proliferation [3–5]. While airway epithelial loss is due to sustained effects of inflammatory mediators [6, 7], the subsequent process of epithelial cell proliferation and restitution is broadly attributed to a subclass of receptor tyrosine kinases (RTK) called the ERbB's [8, 9]. ERbB family of receptors is composed of four members, namely ERbB1, ERbB2, ERbB3 and ERbB4. Phosphorylation of ErbB receptors by ligand binding induces heterodimerization of these receptors and further leads to activation of specific signaling cascades. The ligands for these receptors are epidermal growth factor (EGF) conserved peptide growth factors [10]. In this context, MUC4, an airway mucin with EGF-like domains in its trans-membrane subunit, has been identified as a possible ligand for ERbB2 receptor [11].

Human MUC4 is a high molecular weight membrane-bound mucin that is expressed in goblet and ciliated cells of trachea and bronchus. The gene was originally cloned from a human tracheobronchial cDNA library and was mapped to chromosome 3, at q29 [12]. MUC4 is composed of two subunits, a larger (850 kD) extracellular glycoprotein MUC4 α subunit and a smaller (80 kD) transmembrane MUC4 β subunit characterized by two EGF-like domains [13]. However, recent reports indicate the presence of three EGF-like domains in the MUC4 β subunit [14, 15].

IL-9, a Th₂ lymphocyte-derived cytokine, has been reported to have a variety of functions that include proliferation of T cells, stimulation of IgE production from B lymphocytes [16] and promotion of differentiation of mast cells and hematopoietic progenitor cells [17, 18]. *IL-9* has been implicated as a candidate gene influencing asthma [19] and increased levels of IL-9 and its receptor have been reported in the upper airways of patients with CF [20]. Transgenic mice models over-expressing IL-9 in lung have been demonstrated to induce airway inflammation, mast cell hyperplasia and bronchial hyper-responsiveness [21].

In an earlier study, we demonstrated the effects of secretagogues, such as 8-bromocyclic AMP and neutrophil elastase, on mucin secretions using a non-small cell lung adenocarcinoma cell line, NCI-H650 [22]. Utilizing the same cell line in the present study, we have investigated the effects of IL-9 upon MUC4 gene and glycoprotein expression. We have also determined that IL-9 transcriptionally up-regulates MUC4 mRNA expression and that selective inhibition of JAK3 signaling pathway has substantial effects on IL-9-dependent MUC4 expression.

Methods

Cell culture

Human bronchioalveolar carcinoma cell line NCI-H650 (ATCC, Manassas, VA), was seeded at a density of 1×10^6 cells/well into six-well plates. The cultures were maintained in serum-free ACL-4 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 0.02 mg/ml insulin at 37°C in 5% CO₂ fully humidified air and were sub-cultured twice weekly. Cell viability was periodically determined by trypan blue

exclusion. The test groups upon confluency, were treated in triplicate with varying concentrations (5–100 nM) of human recombinant IL-9 (Sigma–Aldrich, Saint Louis, MO). Control groups were treated with media alone. Some cultures were pretreated with 4-(4'-hydroxy-phenyl) amino-6, 7-dimethoxyquinazoline (WHI-P131) in a concentration range of 20–80 nM or α -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide (AG-490) at 80 and 100 nM for 1 h before treatment with 25 ng/ml of IL-9.

Post-transcriptional mRNA stability experiments were carried out by incubating IL-9 treated and untreated control groups with 5 μ g/ml of actinomycin D (Sigma–Aldrich, Saint Louis, MO) for varying lengths of time. For Western blotting experiments, cultures were initially treated with IL-9 for 2 h, washed and reincubated in fresh medium without IL-9 for 22 h.

Immunohistochemistry

The presence of IL-9 receptor (IL-9R) on the cell surface was established by using a rabbit polyclonal anti-human IL-9R antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA). Harvested cells were initially washed in PBS and fixed in 4% paraformaldehyde for 5 min. After initial blocking in 4% BSA in PBS solution for 45 min, cells were incubated at room temperature in anti-human IL-9R primary antibody at 1:100 dilutions for 1 h. Secondary antibody incubations were performed with Alexafluor (568 nm)-labeled mouse anti-rabbit antibody at 1:250 dilutions (Molecular Probes, Eugene, OR) for 10 min. Cells were counterstained with DAPI (0.2 μ g/ml in PBS) for 2 min after three successive PBS washes. The cells were then visualized using a Zeiss Axioplan 2 microscope equipped with DAPI: rhodamine filters.

RNA extraction and reverse transcription

Total RNA was extracted by RNeasy Mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The DNase digestion of the RNA samples were performed on RNeasy columns using the RNase-free DNase kit supplied by the same manufacturer. Following DNase digestion, total RNA was eluted with 30 μ l DEPC treated water. Integrity of the eluted RNA was confirmed by electrophoresing 5 μ l of total RNA using 1.2% agarose/formaldehyde gels. Reverse transcription (RT) of eluted RNA was performed using random hexamers (6 μ g/ml, final conc.) and Superscript II First Strand Synthesis kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol.

Real-time PCR experiments

Quantification of genes were performed using fluorescent *Taqman* 6-FAM-TAMRA probes on an ABI-Prism 7000 (PE-Applied Biosystems, Foster City, CA). The sequences for *MUC4* primers and *Taqman* probes used in the study are from areas flanking the tandem repeat domains and were sourced from published literature [23]. Endogenous 18S rRNA standards were obtained commercially (Applied Biosystems). Conditions for PCR amplification included 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The optimal concentrations of primers and probes were 900 nM of forward, 300 nM of reverse and 100 nM of probe per reaction. Relative abundance of *MUC4* was determined by $\Delta\Delta C_t$ method using eukaryotic 18S rRNA as endogenous references. Negative controls were performed by omitting the RT step before PCR amplification to verify that the amplified products were from mRNA and did not represent amplification of genomic DNA.

mRNA stability assay

To test the possibility of post-transcriptional stabilization of MUC4 mRNA, actinomycin D chase experiments were performed. Actinomycin D is an antineoplastic antibiotic that complexes with double-stranded DNA and inhibits DNA primed RNA synthesis [24, 25]. In this study, IL-9 stimulated or resting NCI-H650 cells were treated with actinomycin D (5 µg/ml) for 0, 2, 4, 6 and 8 h. For each time point, total RNA was extracted, DNase digested and reverse transcribed following the procedure described above. MUC4 mRNA levels were evaluated by real time PCR.

Nuclear run-on assay

Nuclei from control and treated cells were extracted using the Nuclei Ez Prep isolation kit (Sigma–Aldrich, Saint Louis, MO) following the manufacturer's protocol. Isolated nuclei were layered onto a sucrose cushion (2.1 M sucrose, 5 mM MgAc₂, 1 mM EDTA, 10 mM Tris–HCl, pH 8, 1 mM EGTA, 1 mM spermidine, 1 mM dithiothreitol and 0.1 mM phenylmethylsulphonyl fluoride—PMSF) by centrifugation for 40 min at 16,000 × *g*. Nuclei from treated and control cells were split into two aliquots. One aliquot (+NTP) was incubated for 45 min at 37°C in a solution containing 20% glycerol, 30 mM Tris–HCl, pH 8, 150 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol and 50 U of RNase OUT (Invitrogen, Carlsbad, CA) and ATP, CTP, GTP and UTP at 0.5 mM concentration each. The other aliquot (–NTP) was incubated in the same solution without nucleotides. After incubations, RNA was extracted, reverse transcribed and analyzed by real-time PCR as described above.

Western blotting

Treated and control cells were lysed by sonication on ice in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 5 µg/ml Aprotinin, 5 µg/ml Leupeptin). Equal amounts of cell lysates (50 µg) were resolved separately on 4–20% SDS polyacrylamide gradient gel and transferred to nitrocellulose membranes. The membranes were then blocked by 5% dry milk in Tris-buffered saline (20 mM Tris–HCl, pH 7.2, 150 mM NaCl) for 2 h at room temperature and then incubated with 1:200 diluted human MUC4-specific 1G8 monoclonal antibody (Zymed labs, San Francisco, CA) for 1 h. Secondary antibody incubations were performed with 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG antibody. After incubations, the membranes were treated with HighSignal West Pico chemiluminescent substrate (Pierce Biotech, Rockford, IL) and exposed to BioMax films (Eastman Kodak Co, Rochester, NY) for 1 min. Coomassie blue staining of gels was performed to check for variations in sample loading.

Downstream pathway analysis

To understand the potential role of JAK3 signaling in IL-9 stimulated *MUC4* expression, confluent cultures were pretreated with a JAK3-selective inhibitor 4-(4'-hydroxyphenyl) amino-6, 7-dimethoxyquinazoline (WHI-P131) in a concentration range of 20–80 nM for 1 h before treatment with 25 ng/ml of IL-9. The experiments were repeated using a JAK2 inhibitor α -cyano-(3,4-dihydroxy)-*N*-benzylcinnamide (AG-490) at 80 and 100 nM to ascertain the specificity of JAK3-selective signaling. Following incubations total RNA was isolated, reverse transcribed and analyzed by real-time PCR as described earlier.

Statistical analysis

The data were analyzed using SAS software V.8.2 (SAS Institute, Cary, NC) by parametric Student *t*-tests. Differences were considered significant at $p < 0.05$.

Results

IL-9R expression

The presence of IL-9R mRNA transcripts in NCI-H650 cells was initially established by reverse transcription polymerase chain reaction (RT-PCR) methodology using specific primers [26]. The expected bands of 439 bp for IL-9R and 1,000 bp band for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained on running the amplified products on 1% agarose–ethidium bromide gel (Fig. 1a).

Localization of IL-9R receptor on the cell surface of NCI-H650 cells was also established by immunohistochemistry. IL-9R staining was observed on NCI-H650 cell surface using rabbit polyclonal anti-human IL-9R antibody but was absent in control cells incubated with non-immune rabbit IgG (Fig. 1b, c).

IL-9 modulated MUC4 expression

Confluent cultures of NCI-H650 were treated with IL-9 in the range of 5–100 ng/ml for 1 h and analyzed for *MUC4* by real-time PCR analysis. The results revealed an increasing expression of *MUC4* with IL-9 exposure, reaching maximum expression levels at 25 ng/ml of IL-9 (Fig. 2).

To determine whether IL-9-induced *MUC4* expression is time-dependent, cultures were exposed to 25 ng/ml of IL-9 from 2 to 8 h. *MUC4* mRNA levels increased after 1 h and reached peak expression levels at 2 h (Fig. 3). The levels of *MUC4* expression were approximately 4.5 times higher than those of controls after 2 h exposure of 25 ng/ml IL-9.

Transcriptional regulation of *MUC4*

In order to determine the regulation of *MUC4* expression, nuclear run-on assays were performed. Nuclei extracted from IL-9 treated cells incubated with a mixture of NTP's (Tr(+NTPs)) revealed higher *MUC4* mRNA expression over nuclei from treated cells incubated without NTP's (Tr(–NTPs)). However, no significant difference in *MUC4* transcription levels were observed between nuclei from control cells incubated with NTP's (C(+NTPs)) over those incubated without NTP's (C(–NTPs)) (Fig. 4). The viability and integrity of nuclei from all groups were tested by trypan blue exclusion method on a hemocytometer and were consistently in excess of 95%.

MUC4 mRNA stability

Levels of *MUC4* mRNA in IL-9 treated and non-treated controls steadily decreased with time on exposure to actinomycin D. No significant ($p=0.06$) enhancement in *MUC4* transcript half-life was observed in IL-9 treated cells over sham treated controls. After actinomycin D treatment for ~7.5 h, *MUC4* mRNA concentrations in treated and control groups were half that of their initial levels. The results are plotted assuming initial mRNA levels at 0 h in IL-9

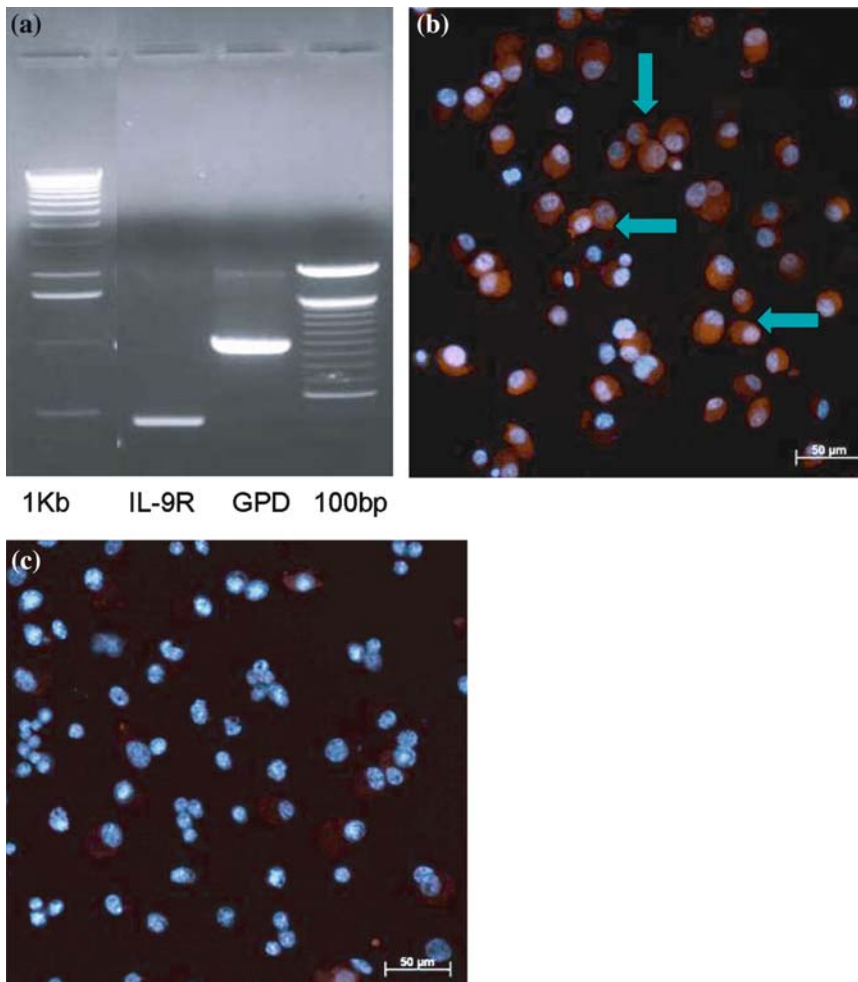


Fig. 1 (a) RT-PCR analysis of IL-9R mRNA expression from NCI-H650 cells. Total RNA was extracted from confluent cultures and analyzed for the presence of IL-9R and GAPDH transcripts by RT-PCR. The amplified products were run on 1% agarose–ethidium bromide gels. Bands at 439 bp (IL-9R) and 1000 bp (GAPDH) were observed. Immunohistochemical localization of IL-9 receptors on NCI-H650 cell surface using (b) rabbit polyclonal anti-human IL-9R antibody (c) and non-immune rabbit IgG serum (control). Secondary antibody incubations were performed with Alexafluor (568 nm)-labeled mouse antibody. Cells were counterstained with DAPI and photographed using dapi: rhodamine filters at 25:2,000 ms exposure. Block arrows in (b) are indicative of specific staining to IL-9 receptors on cell surface

treated and control cells to be 1 (Fig. 5). These experiments indicate that IL-9 does not enhance post-transcriptional MUC4 mRNA stability.

MUC4 glycoprotein expression

MUC4 glycoprotein expression was determined by Western blotting using a 1G8 antibody specific to ASGP-2 region of human MUC4 mucin. Upon IL-9 stimulation, MUC4 levels increased (Fig. 6). A specific band was noted at approximately 147 kDa.

Fig. 2 Dose-dependent expression of *MUC4* by IL-9. NCI-H650 cells were treated with 0–100 ng/ml concentrations of IL-9. The mRNA levels of *MUC4* were determined by real-time PCR analysis. The data are representative of three independent experiments with each treatment run in triplicate. Data were statistically analyzed by student *t* test. * Significantly different, $P < 0.05$

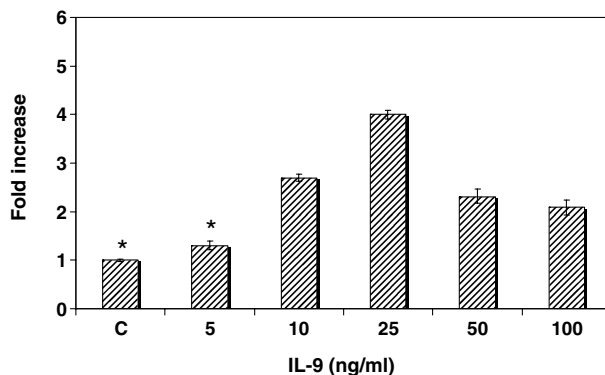


Fig. 3 Time course of *MUC4* gene expression by IL-9. NCI-H650 cells were treated with 25 ng/ml of IL-9 for indicated times. Controls were sham treated. [■] *MUC4* and [□] 18S rRNA eukaryotic mRNA levels were determined by real-time PCR amplification. The graph summarizes data from three independent experiments with each treatment run in triplicate. Statistical analyses were performed by student *t* test. * Significantly different, $P < 0.05$

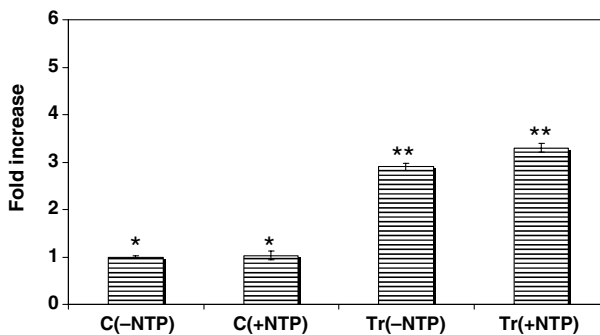
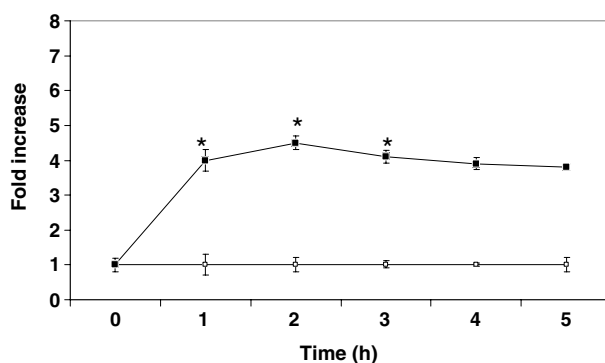


Fig. 4 Transcriptional regulation of *MUC4* by IL-9. Nuclei were isolated from IL-9-treated, control cells, and incubated with or without a mixture of NTP's (0.5 mM each) for 45 min at 37°C. Real-time PCR amplifications were performed on total RNA extracted from: C(-NTP) untreated nuclei of control cells; C(+NTP) NTP treated nuclei of control cells; Tr(-NTP) untreated nuclei from IL-9-treated cells; and Tr(+NTP) NTP treated nuclei from IL-9-treated cells. The graph summarizes data from three independent experiments. Statistical analyses were performed by student *t* test. * Non-significant, $P > 0.05$; ** significantly different, $P < 0.005$

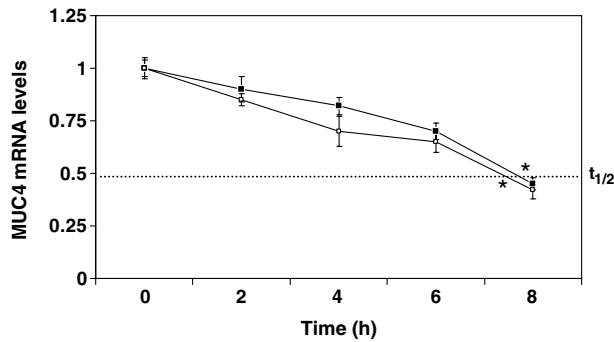


Fig. 5 MUC4 mRNA stability studies following actinomycin D treatments. Cells were treated with or without 25 ng/ml of IL-9 and then exposed to 5 μ g/ml of actinomycin D for varying times. MUC4 data plotted in the graph is representative of three independent experiments from (■) IL-9 treated and (□) untreated control cells. The initial concentrations of MUC4 mRNA at 0 h after actinomycin D treatments were arbitrarily assumed as 1. * No significance was observed in $t_{1/2}$ values of MUC4 in IL-9 treated cells over controls, $P>0.05$

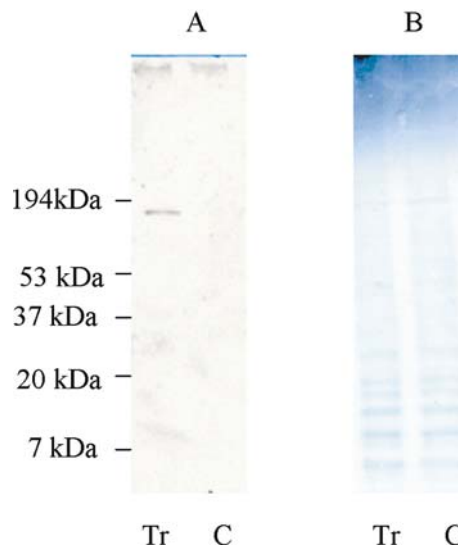
Effects of JAK3 inhibitor on MUC4 expression

NCI-H650 cells at confluency were treated with 20–80 nM concentrations of WHI-P131 for 1 h before treatment with IL-9. The results revealed a systematic down-regulation of MUC4 mRNA levels with increasing concentrations of WHI-P131. At a concentration of 80 nM of WHI-P131, IL-9-induced up-regulation of MUC4 was abolished (Fig. 7). Under similar experimental conditions, pretreatments with AG-490 at 80 and 100 nM concentrations before IL-9 exposure revealed no significant effect on MUC4 regulation (data not shown).

Discussion

In this study, we demonstrated that IL-9-mediated MUC4 gene regulation is dose- and time-dependent and that the regulation is primarily at the transcriptional level in NCI-H650 cells.

Fig. 6 MUC4 glycoprotein expression on IL-9 stimulus. Panel (A) Confluent NCI-H650 cells were treated with (Tr) 25 ng/ml of IL-9 for 2 h followed by a 22 h chase period in fresh medium without IL-9. (C) The control cells were untreated. Total cellular protein (50 μ g) was resolved on 4–20% SDS-PAGE gradient gels and evaluated with 1G8 anti-MUC4 monoclonal primary antibody and horse-radish peroxidase conjugated goat secondary antibody. The Western blots are representative of three separate experiments. Panel (B) Commassie blue staining of the gels



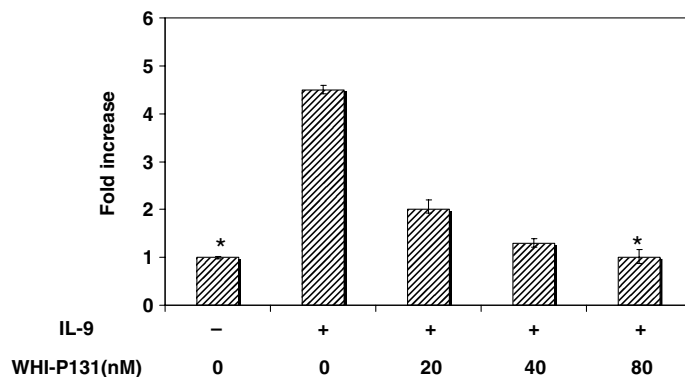


Fig. 7 Effects of JAK3-selective inhibitor, WHI-P131, upon IL-9-stimulated MUC4 mRNA expression. NCI-H650 cells were pretreated with indicated concentrations of WHI-P131 for 1 h before stimulation with 25 ng/ml of IL-9. Quantification of *MUC4* was performed by real time PCR and relative expression determined by $\Delta\Delta C_t$ method. Statistical analyses were performed by student *t* test. * No statistical significance, $P > 0.05$

Elevation of MUC4 glycoprotein levels was also shown by Western blot analysis using a monoclonal antibody specific to the MUC4 β non-tandem repeat region. Further, pretreatment with JAK3-selective inhibitor WHI-P131 decreased IL-9 stimulated *MUC4* expression in a dose-dependent fashion suggesting a possible JAK3 signaling pathway.

Mucus hyper-secretion is a common pathological feature of various chronic inflammatory diseases of the lung. Accordingly, the majority of studies reported earlier were performed to explain the regulatory aspects of inflammatory mediators upon secretory mucin gene expression. Gene expression of airway secretory mucins, such as MUC5AC, MUC2 and MUC8, is regulated by a variety of cytokines and proteolytic enzymes [6]; however ambiguity persists on whether this regulatory pattern is exclusive to few or uniform across all airway mucin genes. For example, IL-4 decreases MUC5AC and increases MUC8 mRNA levels in cultured human nasal epithelial cells [27]; IL-13 was reported to increase *MUC2* and decrease *MUC5AC* expression in culture conditions [28]. Further, the role of these mediators on membrane-bound mucins, such as MUC4, has not yet been defined.

Membrane-bound mucins, such as MUC1 and MUC4, modulate cell–cell and cell–extra cellular matrix interactions by steric hindrance on epithelial cell surfaces [29]. In addition to mechanical functions, MUC1 and MUC4 participate in cellular signaling. For instance, MUC1 associates with β -catenin [30], with Grb2/Sos signaling molecules [31] and with ErbB family of RTK's [32]. On the other hand, MUC4 is the only ligand characterized for ErbB2 receptor activation [33].

To date, several airway epithelial cell lines such as A549, Calu-3, HM3 and H292, have been used as *in vitro* model systems for mucin gene expression studies involving cytokines [34–37], air pollutants [38], bacterial endotoxins [37, 39–41] and serine proteases [42]. In an earlier study, a similar cell line NCI-H650 was demonstrated to secrete mucins in culture conditions by a variety of secretagogues, such as 8-bromocyclic AMP, neutrophil elastase and ionomycin, using a polyclonal antibody to normal human tracheobronchial mucin (HTM-1) [22]. Utilizing the same cell line in this report, we studied the effects of IL-9 upon MUC4 gene and glycoprotein expression. In order, to establish the presence of specific IL-9R receptor in NCI-H650 cells immunohistochemical staining was performed using a rabbit polyclonal antibody specific to human IL-9R. The expression of IL-9R in NCI-H650 cells was further

substantiated by RT-PCR experiments using IL-9R-specific primers, which revealed a 439 bp band consistent with the published literature [26].

In an earlier study, exposure of IL-9 to primary epithelial cells for 4 days, revealed an increase in MUC2 and MUC5AC mRNA levels over sham treated controls [43]. Under the same conditions, no increase in *MUC4* levels was reported based on a semi quantitative RT-PCR methodology. It is possible that sustained exposure of IL-9 to airway epithelial cells results in the release of chemokines, which may indirectly affect *MUC* gene expression. Studies based on IL-9 transgenic mice models and *in vitro* cell systems have revealed that IL-9 induces chemokine [44] and cytokine [45] production in airway epithelial cells. For example, intra-tracheal instillation of murine IL-9 increased the production of IL-13 mRNA after 4 days in the lungs of mice [46]. In murine lung epithelial cell lines, IL-9 exposure for 5 days induced expression of CC chemokines such as eotaxin and MCP-5 [44]. These mediators directly, or in conjunction with IL-9, regulate the expression of a variety of mucin genes [28, 47, 48]; however, the influence of these mediators in this study would be minimal as we detected an increase in *MUC4* mRNA levels after 1 h of IL-9 exposure. These findings reveal a direct effect of IL-9 upon *MUC4* gene expression *in vitro* and are based on quantitative PCR methodology.

Transcriptional regulation of *MUC4* by IL-9 was established by adopting a PCR-based nuclear run-on assay published earlier by Rolfe et al. [49, 50]. These findings are confirmatory of studies where IL-1 β transcriptionally regulated *MUC2* and *MUC5AC* gene expression in H292 cells [51] and TNF- α induced *MUC1* mRNA expression at a transcriptional level in human uterine epithelial cells [52]. On the other hand, these results stand in contrast to reports involving neutrophil elastase (NE), which post-transcriptionally enhanced mRNA stability of *MUC5AC* and *MUC4* genes in A549 and NHBE cells, respectively [42, 53]. Specific mechanisms of NE-mediated mRNA stability are not known. It is reported that NE treatments release secondary mediators that influence overall mRNA stability and expression. For example, NE treatments release TGF- β [54], which has been reported to influence *Muc4*/SMC mRNA stability. These discrepancies suggest that *MUC* gene regulation could, in part, be mediator-specific.

The monoclonal *MUC4* 1G8 antibody used in the study was developed using purified ASGP-2 (rat *MUC4* β) immunogen derived from 13,672 rat mammary adenocarcinoma cells. Specificity of the antibody to human *MUC4* was tested by immunoblotting against recombinant human *MUC4* β expressed in Cos-7 and HC11 murine epithelial cells [55]. Western blotting analysis using 1G8 antibody in this study, revealed a 147 kDa band which is consistent with earlier reports determining *MUC4* expression following neutrophil elastase (NE) exposure in normal human bronchial epithelial (NHBE) cells and similar to *MUC4* β determined in human ocular secretions and corneal epithelium [56].

Of the four members of the mammalian JAK family (JAK1, JAK2, JAK3 and TYK2), tyrosine kinase JAK3 has been shown to undergo marked tyrosine phosphorylation in response to all cytokines that use the common IL-2R, including IL-2, IL-4, IL-7, and IL-9 [57–60]. With this insight, we investigated the possible role of IL-9-stimulated JAK3-specific signaling upon *MUC4* gene expression in NCI-H650 cells. 4-(4'-hydroxyphenyl) amino-6, 7-dimethoxyquinazoline (WHI-P131) is a JAK3-selective inhibitor with no effects on JAK1, JAK2, ZAP/Syk or Src kinases. WHI-P131 was identified as an anti-thrombotic agent that inhibits JAK3 pathway-dependent platelet aggregation [61]. The exposure of NCI-H650 cells to WHI-P131 before IL-9 stimulus down-regulated *MUC4* gene expression in a dose-dependent fashion. To ascertain further the specificity of inhibition, a JAK-2 inhibitor, α -cyano-(3, 4-dihydroxy)-*N*-benzylcinnamide (AG-490) [62], was used under similar experimental conditions. The results indicated no substantial change in IL-9-induced *MUC4* expression. Our results are similar to

earlier results where regulation of *MUC5AC*, a major airway secretory mucin, was reported to be modulated by IL-9 via JAK/STAT pathway [26]. While, the possibility of parallel activation of JAK1 and JAK3 pathways by IL-9 may also exist, this study explored the relevance of JAK3 signaling cascade on *MUC4* gene expression.

While, *MUC4* is expressed in airway epithelium from large airways to bronchioles, its specific utility to overall lung function is not yet properly defined. However, reports indicating the expression of *MUC4* mRNA in branching airways during early fetal lung development [63] and strong induction of *MUC4* in differentiating airway epithelium suggest a possible role in epithelial cell differentiation [64]. Based on studies of *Muc4*/SMC (rat homologue of human *MUC4*) it is hypothesized that *MUC4* on binding to ErbB2 receptor activates downstream signaling which alters cell differentiation and in conjunction with neuregulin stimulates cell proliferation [65].

In a recent study, evaluation of airway biopsies from asthmatic patients by RT-PCR methodology has hinted at low, yet increased *MUC4* protein levels over normal healthy controls [66]. While immune cell derived, cytokines like IL-9 are known to have significant effects on airway inflammation and tissue damage in asthma, this report demonstrates their direct role in regulation of a membrane bound mucin *MUC4* in-vitro.

IL-9 is one among a wide variety of other inflammatory mediators prevalent in chronic lung disease and hence it would be interesting to study how IL-9 interrelates with other cytokine networks localized to lungs in mediating *MUC4* regulation.

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