Regulation and cross-talk between environmental triggers of local immune responses in airway epithelial cells

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Chapter 4
EGR-1 and DUSP-1 are important negative regulators of pro-allergic responses in airway epithelium

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ABSTRACT

Background: Primary nasal epithelium of house dust mite allergic individuals is in a permanently activated inflammatory transcriptional state.

Objective: To investigate whether a deregulated expression of EGR1 and/or DUSP1, two potential negative regulators of pro-inflammatory responses, could contribute to the activation of the inflammatory state.

Methods: We silenced the expression of EGR1 or DUSP1 in the airway epithelial cell line NCI-H292. The cell lines were stimulated in a 24-hour time course with house dust mite allergen or poly(I:C). RNA expression profiles of cytokines were established using q-PCR and protein levels were determined in supernatants with multiplex ELISA.

Results: The shRNA-mediated gene silencing reduced expression levels of EGR1 by 92 % (p<0.0001) and of DUSP1 by 76 % (p<0.0001). Both mutant cells lines showed an increased and prolonged response to the HDM allergen. The mRNA induction of IL-6 was 4.6 fold (p=0.02) and 2.4 fold higher (p=0.01) in the EGR1 and DUSP1 knock-down respectively when compared to the induced levels in the control cell line. For IL-8, the induction levels were 4.6 fold (p=0.01) and 13.0 (p=0.001) fold higher. The outcome was largely similar, yet not identical at the secreted protein levels. Furthermore, steroids were able to suppress the poly(I:C) induced cytokine levels by 70 - 95 %.

Conclusions: Deregulation of EGR-1 and/or DUSP-1 in nasal epithelium could be responsible for the prolonged activated transcriptional state observed in vivo in allergic disease. This could have clinical consequences as cytokine levels after the steroid treatment in EGR1 or DUSP1 knock-down remained higher than in the control cell line.

INTRODUCTION

In addition to the well-described involvement of dendritic cells and lymphocytes in mounting an immune response to allergens, we now also acknowledge the role of airway epithelial cells. Epithelial cells respond to environmental factors such as allergens, bacteria, or viruses by the production of inflammatory mediators through which they may contribute to the modulation of local immune responses [1, 2]. In this manuscript we built on our previous observations that suggest that primary nasal airway epithelial cells from house dust mite allergic individuals are in a permanently activated state [3].

We have previously shown that nasal epithelial cell are able to respond to exposure to the house dust mite (HDM) allergen and that this response is different for epithelial cells isolated from healthy or from allergic individuals [3]. In summary, we found that there is a selective group of genes, such as transcription factors of the NF-κB family (NFkB1, NFkB2, and RELB) and the AP-1 family (JUNB and FOSL1), traditionally linked to pro-inflammatory processes [4], that is strongly up-regulated in epithelial cells from healthy individuals when exposed in vitro to HDM, while these same genes already have a high expression level at baseline in nasal epithelial cells from allergic individuals, which remains permanently high after the allergen challenge. Moreover, another group of genes have a similar low level of expression at baseline in nasal epithelial cells from both allergic individuals and healthy controls and these genes fail to be up-regulated in response to HDM allergen in allergic
subjects, whereas they are up-regulated in healthy controls. Some of the genes belonging to the last group of genes have previously been associated with negative regulation of inflammatory responses (EGR-1 and DUSP-1) [5, 6]. This observation led us to hypothesize that the interplay between these transcription factors could define the allergic state in primary nasal epithelial cells. Additionally, our recent study on the airway epithelial NCI-H292 cell line demonstrated a strong similarity of the responses to HDM and the viral dsRNA analogue poly(I:C), both at the gene expression and at the mediator level [7]. However, whether EGR-1 and DUSP-1 deregulation in an allergy setting can also affect the anti-viral responses remains unexplored.

To explore whether EGR-1 and DUSP-1 can act as negative regulators of allergen induced responses in airway epithelial cells, we used the NCI-H292 cell line model that we have previously investigated for its response to allergens in great detail [8]. As part of that work we have shown that this cell line shares a core HDM allergen response with primary healthy nasal epithelial cells that comprises the pro-inflammatory transcription factors (NFKB1, NFKB2, and JUN) and the potential negative regulators EGR-1 and DUSP-1. In the current manuscript we show that knocking down the expression of either EGR-1 or DUSP-1 leads to an increased induction of the pro-inflammatory cytokines IL1-RA, IL-6, IL-7, IL-8, and VEGF after HDM allergen exposure as compared to the wild type cell line. A similar, yet not identical, outcome was seen after stimulation of the mutant cell lines with poly(I:C), strengthening our previous idea of a conserved mechanism that links anti-viral responses to the responses induced by allergens [8]. Moreover, here we report on a potential clinical consequence as we show a reduced effectiveness of steroid treatment due to increased induction levels of pro-inflammatory mediators in the EGR-1 and DUSP-1 mutant cell lines.

MATERIALS AND METHODS

Cell cultures
NCI-H292 human airway epithelial cells (American Type Culture Collection, USA) were cultured in RPMI 1640 culture medium (Invitrogen, NL) supplemented with 10% (v/v) fetal calf serum (HyClone, USA), 1.25 mM of glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin. Cells were grown in fully humidified air containing 5% of CO₂ at 37°C. The EGR-1 knock down or DUSP-1 knock down mutant cell lines growth medium was additionally supplemented with 2 µg/mL of puromycin (Sigma-Aldrich, NL).

Silencing of EGR-1 or DUSP-1 gene in NCI-H292
Silencing of the EGR-1 or DUSP-1 gene in the H292 cell line was achieved by lentivirus mediated short hairpin RNA expression using shRNA plasmids obtained from Sigma-Aldrich (NL). HEK 293T cells were transfected with vectors necessary for virus assembly: pMDLg/pRRE, pRSV REV, and pMD2.G together with shRNA expressing vectors that target EGR-1, DUSP-1 or irrelevant RNA and carry the puromycin resistance vectors pLKO-EGR.1.4 or pLKO-DUSP.1.4, or pLKO-NT.2 in presence of Fugene HD (Promega, NL) for 12 hours. Lentiviruses produced by the 293T cells and carrying the EGR-1 or DUSP-1, or irrelevant RNA targeting vectors were collected by 60 minutes ultracentrifugation of 48 hours supernatants. Stable
EGR-1, DUSP-1, or irrelevant-RNA shRNA targeted NCI-H292 cell lines were generated by 24 hours incubation of the parent NCI-H292 cells with appropriate lentiviruses followed by 10 days selection of puromycin resistant NCI-H292 mutant cells. Gene silencing efficiency was confirmed by quantitative PCR.

**Experimental set-up**

NCI-H292-mutant cells (EGR-1 knock-down, DUSP-1 knock-down, and non-targeted control strain) were cultured to 80% confluence in 6- or 12-well plates and prior to stimulation the culture medium was removed and replaced with serum-free RPMI. Cells were then stimulated with 45 µg/mL of HDM (kindly provided by HAL Allergy, NL) diluted in RPMI medium or with 20 µg/mL of polyinosinic:polycytidylic acid [poly(I:C)] (Sigma-Aldrich, NL) in a time course over 24 hours. 15, 30, 60, 120, 240 minutes, 8, 16, and 24 hours after stimulation supernatants were removed and those of 8 and 24 hours were stored at -20°C for further analysis. Cells were harvested by application of 1 mL of TRIzol (Life Technologies, USA) onto a well and stored at -20°C for RNA and protein isolation.

In the experiment with steroid, cells were stimulated with 20 µg/mL of poly(I:C) in the presence of 10⁻⁷ M of dexamethasone (Sigma-Aldrich, DE) for 8 hours. Cell-free supernatants were collected and stored at -20°C until further analysis.

The data shown is from one representative experiment (out of three) and each value represents an average value of three biological replicates with a standard deviation.

**RNA extraction**

The total RNA was extracted by TRIzol (Life Technologies, USA) and chloroform (Merck, DE) phase separation method and purified with nucleospin RNA II kit (Machery-Nagel, DE). RNA quality was checked on the Agilent 2100 bio-analyzer (Agilent Technologies, USA).

**Quantitative PCR**

The MBI Fermentas first strand cDNA synthesis kit (Thermo Scientific, NL) was used for cDNA reverse transcription. Real-time PCR was performed in Bio-Rad iCycler (Bio-Rad, NL) with mRNA specific TaqMan gene expression assays (Applied Biosystems, NL) for the EGR-1 (HS00152928_M1) and DUSP-1 (HS00610257_G1) according to the manufacturer’s protocol or with IQ™ SYBR Green Supermix (Bio-Rad, NL) with the following primers: GAPDH-forward: 5’-GAAGGTGAAGGTCGGAGTC-3’; GAPDH-reverse: 5’-GAAGATGGTGATGGGATTTC-3’; IL-6-forward: 5’-TGACAAACATCCTGATGAGATC-3’; IL-6-reverse: 5’-AGTGCCTCTTTTGCTTTTCAC-3’; IL-8-forward: CCACACTGCGCCAACACAGAAATTATTG-3’; IL-8-reverse: 5’-GCCTCTTTCAAAAACCTTCCACAAAACC-3’.

Data was analyzed in the Bio-Rad CFX Manager program (Bio-Rad, NL) and fold changes of evaluated genes were calculated using the comparative ∆∆Ct method. Each value was corrected for the expression of the housekeeping gene and compared to the control condition (cell exposure to resting medium without stimuli). Statistical significance (p<0.05) was determined unpaired Student’s t test using SPSS Statistics (IBM) for Windows.

**Determination of cytokine and chemokine production by ELISA**

Measurements of secreted cytokines were performed by sandwich ELISA in 24-hour cell-free supernatants. The release IL-6 and IL-8 was detected using pairs of specific
monoclonal antibodies and recombinant standards obtained from BioSource International (Camarillo, USA). Statistical significance (p<0.05) was determined using unpaired Student’s t test using SPSS Statistics (IBM) for Windows.

**Protein multiplex ELISA**

Cells-free supernatants of 24 hours HDM and poly(I:C) stimulated NCI-H292 cells (control cell line, EGR-1-, and DUSP-1 knock down cell lines) together with non-stimulated RPMI 1640 controls were used to determine protein levels of the following mediators: IL-1RA, IL-1β, IL-2R, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, TNF-α, IFN-α, IFN-γ, MCP-1, GM-CSF, G-SCF, VEGF, FGFB-β, EGF, HGF, MIG, RANTES, MIP1-α, MIP1-β, and IP-10. Cytokine levels were measured with a use of a Human Cytokine Thirty-Plex Antibody Bead Kit (Life Technologies, NL) in combination with a Bio-Plex workstation (Bio-Rad, NL). All standards were diluted in RPMI 1640 medium as described by the manufacturer. Luminex software was used for the protein concentration calculations and all concentrations are expressed in pg/mL. Statistical significance (p < 0.05) was determined with t test using SPSS Statistics (IBM) for Windows.

**RESULTS**

**Silencing of EGR-1 or DUSP-1 resulted in reduction of target genes mRNA levels**

Targeted gene knock-down resulted in a reduction of basal EGR-1 gene expression by 92 ± 2 % (p < 0.0001), when compared to the EGR-1 gene expression level in the non-targeted control strain and by 76 ± 6 % (p < 0.0001) of DUSP-1 (Figure 1). Moreover, the basal expression of EGR-1 and DUSP-1 was not affected in the non-targeted control, while silencing of EGR-1 did not affect the expression of DUSP-1 and conversely, DUSP-1 silencing did not have any effect on EGR-1 expression level.

![Figure 1. Silencing efficiency of the EGR1 and DUSP1 genes in the NCI-H292 cell line.](image)

**Figure 1. Silencing efficiency of the EGR1 and DUSP1 genes in the NCI-H292 cell line.** shRNA-mediated gene silencing in H292. EGR1 and DUSP1 gene expression levels in the EGR1 knock-down cell line and EGR1 and DUSP1 gene expression levels in the DUSP1 knock-down cell line. Levels of the target genes expression in the control strain were normalized to 100. Changes in the gene expression levels were considered significant if p < 0.05 (*).

**EGR-1 or DUSP-1 knock-down results in an enhanced up-regulation of IL-6 and IL-8 mRNA expression after HDM allergen exposure**

HDM allergen exposure in a time course over 24 hours revealed a prolonged and enhanced up-regulation of IL-6 and IL-8 expression in the EGR-1 and DUSP-1 knock-down cell lines. In the control cells, the maximal up-regulation of 2.7 ± 0.1 for

EGR-1 and DUSP-1 are negative regulators of pro-allergic responses in airway epithelium.
IL-6 (p = 0.006) and 5.2 ± 0.2 fold (p = 0.006) for IL-8 was reached 8 hours after stimulation and gradually returned to their baseline expression level 24 hours after the challenge (Figure 2A and B). The cell line, with the EGR-1 gene silenced, responded more rapidly to HDM and reached significant values of up-regulation already at 2 hours for IL-6 (18.5 ± 0.3 fold, p < 0.0001) and at 4 hours for IL-8 (5.5 ± 0.1 fold, p = 0.01). The maximal expression level of IL-6 was observed at 4 hours (19.0 fold ± 0.3, p < 0.0001) and at 8 hours for IL-8 (11.8 ± 1.2 fold, p = 0.02). These induction values in the EGR-1 mutant are significantly higher (p = 0.008 for IL-6 and p = 0.02 for IL-8) when compared to the induction levels achieved in the non-targeted control at 4 hours for IL-6 (2.1 ± 0.1 fold, p = 0.02) and at 8 hours for IL-8 (5.2 ± 0.2 fold, p = 0.005). As a consequence, the area under the curve, which is a measure of the total amount of mRNA produced after HDM induction, is 5.8 ± 0.4 fold higher for IL-6 (p = 0.001) and 2.4 ± 0.3 fold higher for IL-8 (p = 0.01) in the EGR-1 knock-down compared to the non-targeted control.

Figure 2. EGR1-, DUSP1- knock-down, and non-targeted control NCI-H292 strains were exposed to HDM. Gene expression levels of IL6 (A) and IL8 (B) were quantified by Real-Time PCR in a time course over 24 hours. IL-6 (C) and IL-8 (D) protein levels were measured in cell free supernatants of 8 and 24 hours. Statistically significant (p < 0.05) enhancement of IL6 and IL8 up-regulation levels or IL-6 and IL-8 production rates in the EGR1 or DUSP1 knock-down cell lines compared to the non-targeted control strain are indicated (*).

These observations were mirrored in the DUSP-1 knock-down cell line. Here, HDM allergen challenge induced a rapid and significantly higher than in the control cell line up-regulation of IL-6 seen again already 2 hours after the challenge (8.5 ± 0.1 fold, p < 0.0001) reaching its maximal induction 8 hours after the stimulation (9.0 ± 0.5 fold, p = 0.001). DUSP-1 silencing contributed also to a dramatic up-regulation of IL-8 with a 83.0 ± 0.1 (p < 0.0001) fold induction level 2 hours after HDM stimulation, which is almost 20 times more than in the control cells. Not only were the IL-6 and IL-8 genes super up-regulated in the DUSP-1 knock-down cell line, but also their induction was prolonged over the 24 hour time course. 24 hours after the stimulation, the expression levels of IL-6 and IL-8 were still 6.4 ± 0.3 (p = 0.015) and 12.1 ± 0.4 fold (p = 0.005) up-regulated, whereas in the control cells and in the EGR-
1 knock-down cell line, these genes already had returned to their baseline levels. Consequently, the area under the expression curve is 4.6 ± 0.3 fold higher for IL-6 ($p = 0.02$) and 13.0 ± 0.9 fold for IL-8 ($p = 0.001$) in the DUSP-1 mutant when compared to the control strain.

**DUSP-1 and EGR-1 knock-down enhance IL-6 and IL-8 protein production in NCI-H292**

Figures 2C and 2D show rapid and significant enhancement of the production and release of mediators in response to HDM in the DUSP-1 knock-down cell line. Rapid induction of cytokines production at 8 hours led to 3.6 ± 0.2 fold ($p = 0.01$) and 10.5 ± 1.1 ($p = 0.005$) fold super up-regulation of IL-6 and IL-8 production by DUSP-1 knock-down cells when compared to the induced control cell line production level. 24 hours stimulation of the DUSP-1 knock-down cells with HDM showed a similar enhancement of IL-6 and IL-8 release (4.0 ± 0.2 fold, $p = 0.01$ and 10.1 ± 1.1 fold, $p = 0.015$). Also EGR-1 silencing led to increased production levels of IL-6 and IL-8 at 24 hours compared to non-targeted control, although these levels were not as high as in the DUSP-1 knock-down cell line. 24 hours cell exposure to HDM resulted in 1.5 ± 0.1 ($p = 0.04$) fold enhancement of induction of IL-6 release and 6.7 ± 1.1 ($p = 0.03$) fold of IL-8, while 8 hours of HDM exposure did lead to a super-induction of IL-8 production (1.7 ± 0.3 fold, $p = 0.05$), but IL-6 production levels remained similar when compared to the control cells.

![Figure 3](image-url)

**Figure 3.** *EGR1*, *DUSP1* knock-down, and non-targeted control NCI-H292 strains were exposed to poly(I:C). Gene expression levels of *IL6* (A) and *IL8* (B) were quantified by Real-Time PCR in a time course over 24 hours. IL-6 (C) and IL-8 (D) protein levels were measured in cell free supernatants of 8 and 24 hours. Statistically significant ($p < 0.05$) enhancement of *IL6* and *IL8* up-regulation levels or IL-6 and IL-8 production rates in the *EGR1* or *DUSP1* knock-down cell lines compared to the non-targeted control strain are indicated (*).
DUSP-1 and EGR-1 knock-down also affect IL-6 and IL-8 after poly(I:C) induction

Stimulation of the mutant cell lines with poly(I:C) in a 24-hour time course revealed that, at the mRNA level, only the DUSP-1 knock-down cell line contributed to the enhanced induction of the IL-6 and IL-8 genes (Figure 3A and B, respectively) with an increase in the area under the curve of 1.4 ± 0.2 fold for IL-6 (p = 0.04) and 1.7 ± 0.1 fold for IL-8 (p = 0.01). The data for the amount of IL-6 and IL-8 protein secreted after induction by poly(I:C) in the DUSP-1 mutant mirrors the mRNA expression profile.

The increased expression of IL-6 mRNA at 8 hours that reaches its maximal level at 16 hours (Figure 3A, 1.9 ± 0.2 fold, p = 0.001) also resulted in an increased level of secreted IL-6 protein (Figure 3C). Similarly, the early maximal induction of the IL-8 mRNA at 8 hours (Figure 3B) resulted in a 2.6 ± 0.5 fold (p = 0.04) increased release of IL-8 into the medium after 8 hours, that did not maintain statistical significance at 24 hours (Figure 3D). Moreover, the EGR-1 knock-down cell line, despite that we could not detect any statistically significant changes at the mRNA level, did contribute to significant enhancement of IL-6 and IL-8 protein release at 8 hours (2.7 ± 0.3 fold, p = 0.01 and 4.5 ± 0.4 fold, p = 0.001), and 24 hours challenge (1.6 ± 0.2 fold, p = 0.03 and 1.7 ± 0.3 fold, p = 0.001).

DUSP-1 and EGR-1 knock-down affect the production and release of other pro-inflammatory mediators

To further explore the contribution of EGR-1 and DUSP-1 to the composition of a local tissue microenvironment, we analyzed the production and release level of other cytokines, chemokines, and mediators by cells exposed for 24 hours to HDM or poly(I:C).

There are no statistically significant differences in the baseline production rates of any of the mediators that we were able to detect in the cell-free supernatants (IL1-RA, IL-7, IL-15, IP-10, MCP-1, VEGF, FGF-basic, and HGF) between the non-targeted control strain, EGR-1 knock-down, and DUSP-1 knock-down strains (table 1). Production and secretion of four mediators, namely IL-7, MCP-1, VEGF, and IL1-RA was significantly enhanced by both EGR-1 or DUSP-1 knock-down strains (p < 0.01). Interestingly, the level of VEGF release upon HDM stimulation is significant only in the mutant strains (p < 0.05), whereas its production level by the non-targeted control strain is not affected by the allergen challenge.

The data reveal that the specificity in the contribution of the EGR-1 or DUSP-1 transcription factors to deregulated cell responses is even more pronounced after screening multiple mediators. Production and secretion of IP-10 and FGF-beta after poly(I:C) challenge is enhanced (p < 0.05) only in the DUSP-1 knock-down cells. Even more complex picture arises for hepatocyte growth factor (HGF). HDM-triggered HGF production is enhanced by the EGR-1 knock-down only (p = 0.04), whereas DUSP-1 knock-down strain challenged with poly(I:C) produces significantly more HGF (p = 0.05) than the EGR-1 knock-down or the control strain.

The knock-down strains may also reduce some aspects of cell responses. HDM-triggered production of HGF is down-regulated in the DUSP-1 knock-down cell if compared to the control strain (p < 0.05). IL-15 falls in to the same category. Poly(I:C) stimulation of both mutant strains results in a down-regulation of IL-15 release if compared to the non-targeted control strain (p < 0.01).
Reduced effectiveness of dexamethasone in the EGR-1 and DUSP-1 knock-down cells

Next we investigated whether the increased reactivity to HDM allergen and poly(I:C) in the EGR-1 or DUSP-1 mutant cell lines could have potential consequences for the effectiveness of anti-inflammatory medications.

The power of 10^{-7} M dexamethasone in the reduction of cytokines production in response to poly(I:C) challenge is similar between the control cell line and the EGR-1 or DUSP-1 knock-down cell lines. When expressed as a percentage, the reduction rates by the steroid treatment of IL-6 (92.1 ± 3.0 % for the EGR-1 knock-down and 85.0 ± 9.1% for the DUSP-1 knock-down) and of IL-8 (90.5 ± 8.2 % for the EGR-1 knock-down and 70.3 ± 15.3 % for the DUSP-1 knock-down) are not significantly different than those in the non-targeted control (95.0 ± 19.1 % and 78.2 ± 6.2 % respectively).

However, when we compare the absolute levels of IL-6 and IL-8, a different picture emerges. First of all, the remaining levels of IL-6 and IL-8 in the presence of 10^{-7} M dexamethasone are higher than the corresponding levels in the non-targeted control (figure 4). IL-6 levels are 5.1 ± 0.2 fold higher (p < 0.0001) for EGR-1 and 8.0 ± 1.7 fold higher (p = 0.02) for the DUSP-1 knock-down and IL-8 levels are 1.60 ± 0.2 fold higher (p = 0.03) for EGR-1 and 4.3 ± 1.9 fold higher (p = 0.04) for the DUSP-1 knock-down. As a consequence, the level of expression of IL-8 (54.1 ± 14.1 pg/mL) in the DUSP-1 knock-down in the presence of 10^{-7} M dexamethasone is as high as the expression in the non-targeted control (57 ± 1 pg/mL) in the absence of 10^{-7} M dexamethasone.

**DISCUSSION**

In this manuscript we show that dual specificity protein phosphatase 1 (DUSP-1) and early growth response protein 1 (EGR-1) transcription factors act as independent negative regulators of HDM and a viral analogue poly(I:C) induced gene expression and/or protein production in the airway epithelial cell line NCI-H292. We hypothesize that the deregulated expression of these negative regulators, which we have described in primary nasal epithelium from HDM-allergic individuals [3], could play a role in maintaining the activated transcriptional state of these cells.

Inflammatory cell responses are normally under a tight control and this often include an induction of a negative regulator so that an undesired escalation of an inflammation can be prevented. Both HDM and poly(I:C) induce the expression of the negative regulators EGR-1 and DUSP-1 [3, 9] and now we have shown that EGR-1 and DUSP-1 are negative regulators of pro-allergic responses in airway epithelium.
and DUSP-1 contribute to the inhibition of cell responses to an allergen and to a virus. This would also further support our previous observations that revealed a generalized overlap between allergen and viral induced responses [7]. EGR-1 and DUSP-1 can also be up-regulated by a variety of other stimuli representing both internal and external cell stress factors that may include pathogens/microorganisms, temperature, hypoxia, oxidative stress, or inflammatory mediators [10, 11]. Deregrulated expression of EGR-1 and DUSP-1 has also been linked to allergy-associated processes, such as cell adhesion [12], cell/tissue repair [13], wound healing [14], control of protease inhibitors expression [15], immune modulation [16] or cell recruitment [17]. Consequently, it does not come as a surprise that these transcription factors may also play an important role in an allergic setting.

The main role of DUSP-1 is dephosphorylation of MAP kinase, whereas EGR-1 is a well-known zinc-finger transcription factor [18]. As these two proteins control different steps of a trigger-initiated signaling cascade (f. e. by poly(I:C) or HDM) with DUSP-1 being more upstream of the potential effects of EGR-1 action, it may perhaps partly explain why our data reveals some specificity in the mechanism of the effect of EGR-1 and DUSP-1. After HDM stimulation, the EGR-1 mutant showed higher IL-6 than IL-8 mRNA levels, while the converse was true for the DUSP-1 mutant. Moreover, the response in the DUSP-1 mutant was more prolonged compared to the EGR-1 mutant, independent of the absolute maximal induction levels. The results in the poly(I:C) induction experiment showed that the EGR-1 mutant affected secreted IL-6 and IL-8 protein levels, without affecting their mRNA levels. Indeed, the production and release of functional IL-6 and/or IL-8 may be controlled at many levels, among others by stimulus-dependent transcriptional regulation [19, 20] mRNA stability [21], level of translation [22] or efficiency of secretion [23]. However, the precise mechanism behind the control of IL-6 and IL-8 expression and production in our settings remains unclear. We screened the promoter regions and regulatory elements of the effector molecules, but no (putative) binding sites for EGR-1 and DUSP-1 could be found, which is partially confirmed by others [24]. Given the EGR-1 binding sites identified within the promoter of NFkB1 and NFKB2, and involvement of the DUSP-1 in the basic cell signaling pathways, an indirect effect of EGR-1 and DUSP-1 on the regulation of pro-inflammatory cytokines/mediators seems the most logical.

Transcription factors often form active homo- or heterodimers and their function may strongly depend on the final composition of the complex [25]. Therefore, formation of alternative heterodimers with, e.g. AP-1 family members may not only modulate the affinity of the novel complex to the DNA promoter sequence but also lead to a change of the DNA-strand shape, preventing the transcription factor machinery from assembly at the promoter region [26].

The stimuli that induce EGR-1 or DUSP-1 in many cases are also capable of inducing NF-κB expression and many cytokines/mediators that are relevant for Th1, Th2, or Th17/22 responses are under transcriptional control of NF-κB. The cooperation between NF-κB and EGR-1 may either prevent the NF-κB from binding to the promoter region of a target gene, therefore reduce its expression or may synergistically stimulate the binding frequency to target promoters, hence up-regulate the gene expression. EGR-1 itself possesses the DNA binding capability and several reports have demonstrated a direct induction of genes expression by EGR-1.

Depending on the cell type, trigger, and a duration of the exposure to a stimulus, EGR-1 may act as a suppressor or activator of transcriptional gene expression. That in turn can result in either promotion or inhibition of inflammatory
molecules expression. In T cells, but also in other immune cells, the role of EGR-1 seems to be skewed towards the activation of the pro-inflammatory mode. Expression of the archetypal Th2 cytokine IL-4 is linked with a direct interaction between EGR-1 and IL-4 promoter in T cells [27], mast cells [28], and B cells [29]. Decker and colleagues reported that the interaction between EGR-1 and NF-AT is crucial for IL-2 and TNF-α expression [30, 31] and other pro-inflammatory cytokines expression in T cells [30]. The indirect control of gene expression by EGR-1 may also be associated with gene expression suppression. For instance, EGR-1 has been implicated to directly activate the expression of regulatory miRNA molecules that in turn post-transcriptionally control the level of anti-inflammatory IL-10 production [32] and therefore EGR-1 promotes the expression of pro-inflammatory cytokines in T cells even further.

In contrary to these observations, EGR-1 has been brought in connection of its ability to suppress gene expression, for instance Chapman et al. demonstrated in vitro binding of EGR-1 to RelA and, as a result, inhibition of NF-κB activity [33] and negative feedback loop for EGR-1 and AP-1 has been implicated in dampening of the expression of inflammatory genes [26]. The dual function of EGR-1 may perhaps be related to the competition with the Sp1 transcription factor for the binding site of the target gene [34].

Our data also demonstrate the impact of EGR-1 or DUSP-1 gene silencing on the efficacy of dexamethasone. The enhanced production levels of the mediators in mutant cell lines remain high, even after the dexamethasone co-exposure. Our observations are in line with other reports, for instance, in a DUSP-1−/− mouse model, LPS-induced TNF-α and IL-1β production in BMMs was no longer inhibited by addition of dexamethasone suggesting that, at least partially, the suppressive effect of the steroid treatment is DUSP-1 dependent [35]. Moreover, the action of corticosteroid-mediated dampening of IL-8 in airway epithelium has been associated with an overexpression of DUSP-1 [5, 36].

The NCI-H292 cell line is the model we have extensively used to study human airway epithelium interactions with allergens and viral dsRNA [8, 37] and although we have shown similarities between the responses in NCI-H292 and primary nasal epithelium, we also are aware that the cell line cannot be used as a detailed model of all responses of primary nasal epithelium [8]. We have not been able to knock down the expression of EGR-1 or DUSP-1 to satisfactory rates in human primary airway epithelium and successful knock downs of these transcription factors in primary human airway epithelium, to our knowledge, have never been shown by others. However, the previously described core response to HDM allergen does contain both the pro-inflammatory transcription factors of the NF-κB and AP-1 family as well as the regulators EGR-1 and DUSP-1 so that the data we have obtained in this manuscript are likely also valid in primary nasal epithelial cell.

The mutant cell lines do have some remaining EGR-1 and DUSP-1 activity and can therefore not be compared to mouse lines where the genes have been completely knocked out [38, 39] and are possibly more similar to the situation in human allergic individuals that will have hampered expression rather than missing expression [3]. Whether our observations can be directly translated into clinical implications is unclear given the limited number of mediators we have studied and potential discrepancies between our model and primary airway epithelial cells.

From a clinical point of view, allergic individuals respond to the exposure to an allergen, while non-allergic individuals do not. However, at the molecular level, airway epithelial cells from both allergic and non-allergic individuals do respond to an
allergen challenge and we now show the potential impact of a deregulated expression of EGR-1 and DUSP-1. The increased IL1-RA, IL-6, IL-7, IL-8, MCP-1, VEGF, and partially IP-10 and FGF-basic responses and the down-regulation of IL-15 that we have observed in these mutants can have a big impact through their ability to modulate adaptive immune responses [40, 41]. Understanding why the EGR-1 and DUSP-1 fail to be up-regulated in allergic individuals may contribute to new treatment options. Reduction of the activated state in allergic epithelium by forced up-regulation of these deregulated negative regulators may have direct consequences for the manifestations of allergic symptoms or even in the natural progression of the disease.

<table>
<thead>
<tr>
<th>mediator</th>
<th>baseline</th>
<th>HDM</th>
<th>poly(I:C)</th>
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<tr>
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<td>SD</td>
<td>pg/mL</td>
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Table 1. Mediators secreted after 24-hour cell exposure to HDM or poly(I:C) (non-targeting control cell line; EGR-1 knock-down, or DUSP-1 knock-down). Differences between the knock-downs and the control strain responses to HDM or poly(I:C) were considered significant if \( p < 0.05 \) and are marked with (*).
EGR-1 and DUSP-1 are negative regulators of pro-allergic responses in airway epithelium


EGR-1 and DUSP-1 are negative regulators of pro-allergic responses in airway epithelium.