Nasal epithelial cells: effector cells in allergy
Vroling, A.B.

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A strongly reduced synergistic response to TNF-α and IL-17 detected for the Th1 cytokine INF-γ in primary nasal epithelial cells from allergic individuals.

Aram B. Vroling\textsuperscript{a}, Annemieke Snoek\textsuperscript{b}, Silvia Luiten\textsuperscript{a}, Wytske J. Fokkens\textsuperscript{a}, Rene Lutter\textsuperscript{b}, Cornelis M. van Drunen\textsuperscript{a}

\textsuperscript{a}Department of Otorhinolaryngology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
\textsuperscript{b}Department of Respiratory Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

Submitted
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Abstract

Airway epithelial cells are known to respond to TNF-α by producing a variety of other cytokines in response. For some cytokines this response is enhanced by addition of IL-17. To investigate if the described synergistic effect in lung epithelial cells by simultaneous stimulation with TNF-α and IL-17 can also be observed in primary nasal epithelial cells and to see if there are differences between nasal epithelial cells of allergic and healthy individuals. We cultured primary nasal epithelial cells from healthy and allergic individuals. Upon stimulation we measured cytokine production of 27 mediators. We found synergistic effects on production of IL-6, IL-8 G-CSF and INF-γ. For INF-γ however hits synergistic effect was strongest, but only in epithelial cells of healthy controls. This deregulated response of IFN-γ production in allergic individuals could contribute to disease symptoms, and could play a role in reduced antiviral response observed in allergic individuals.
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Introduction

Previously we have shown that the *in vitro* expression profile in nasal epithelial cells of house dust mite allergic individuals is markedly different from the expression profile in healthy controls. At baseline, in the absence of house dust mite allergen, the differences between allergic and healthy nasal epithelium are best described as a sustained activated state in allergic individuals. Nasal epithelial cells from healthy individuals only reach a similar expression level after *in vitro* exposure to the allergen. Evidently, the allergic status of an individual leaves an imprint on the nasal epithelium which is maintained even after *in vitro* culture in the absence of house dust mite extract. We have hypothesized that a deregulated expression of a group of transcription factors (NF-κB, AP1, ATF3, and EGR1) underlies this activated state and that this state could be maintained by an autocrine loop involving TNF-α. The latter idea was based on the observation that many of the genes that are up-regulated in nasal epithelial after allergen exposure have been reported to be under the transcriptional control of TNF-α. Furthermore, TNF-α has been reported to be a principle downstream target of NF-κB.

Here we analyze the potential role of TNF-α on the activation of nasal epithelial cells in greater detail. Van den Berg *et al* showed that TNF-α-induced IL-6 and IL-8 responses are potentiated by the further addition of IL-17. IL-17 acts as proinflammatory mediator and there is clinical and experimental proof that implicates it in various inflammatory diseases. Increased levels of IL-17 have been reported in allergic asthma, reumathoid arthritis and inflammatory bowel disease. Where TNF-α has a direct effect on the *de novo* synthesis of the mRNAs for these genes, it was suggested that the potentiating effect of IL-17 is mainly through the inhibition of the breakdown of these mRNAs. In this manuscript we analyse the effect of the simultaneous addition of TNF-α and IL-17 to primary nasal epithelial cells from healthy and allergic individuals. For 27 soluble mediators we compare
the effect of the single exposure to either TNF-α or IL-17 to the effect on the combined exposure, and show a strongly reduced synergistic response in nasal epithelium from allergic individuals compared the healthy controls for the Th1 cytokine IFN-γ.

Material and methods

Patient characteristics.

This study was reviewed and approved by the medical ethical committee of the Amsterdam Medical Center and all participants read and signed an informed consent. Five allergic volunteers and five healthy non-smoking volunteers were selected based on skin prick test for house dust mite (HDM) and other common allergens, and a nasal allergen provocation to assess their response. Only monotypically HDM allergic and non-allergic volunteers were included. Allergic individuals had refrained from using any medication for their allergy in the four weeks prior to the visit when biopsies were taken. Biopsies were taken from the lower edge of the inferior turbinate, 1 and 2 cm from the anterior end, using Fokkens’ forceps with a cup diameter of 2.5 mm. Local anaesthesia was achieved by application of adrenalin and cocaine under the inferior turbinate without touching the biopsy site, during 10 minutes.

Primary epithelial cell culture.

Primary cells were obtained by digesting nasal biopsies of volunteers with 0.5 mg/mL collagenase 4 (Worthington Biochemical Corp., Lakewood, NJ) for 1 hour in Hanks’ balanced salt solution (HBSS; Sigma-Aldrich, Zwijndrecht, The Netherlands). Subsequently cells were washed with HBSS and resuspended in bronchial epithelial growth medium (Lonza Clonetics, Breda, The Netherlands) and seeded in a T25 flask and grown in fully humidified air containing 5% CO2 at 37°C, cells were used between passages 3 and 5 until they reached approximately 80% confluency.

Stimulation experiment
Cells were seeded at an initial density of $2 \times 10^4$ in 48-well plates and used between passage 3 and 5 for the 24 hours induction experiment. To assess if simultaneous addition of 5 ng/mL TNF-$\alpha$ and 10 ng/mL IL-17 to nasal epithelial cells has a synergistic effect over addition of either stimulus alone, we calculated the ratio of the expression level of 27 soluble mediators after the simultaneous addition of TNF-$\alpha$ and IL-17, over the sum of the expression level for TNF-$\alpha$ and IL-17 alone (Synergy Factor = $\frac{[\text{mediator}]_{\text{TNF+IL17}}}{[\text{mediator}]_{\text{TNF}} + [\text{mediator}]_{\text{IL17}}}$).

*Luminex assay.*

Supernatants of cytokine stimulated cells were stored at -20°C until analysis. Cytokine levels in supernatant of cells were determined using the xMAP technology (Luminex Corporation, Austin, TX, USA). A Bio-Plex Human Cytokine 27-Plex Panel kit (Bio-Rad, Veenendaal, The Netherlands) was used and analyzed on the Bio-Plex workstation (Bio-Rad). All standards were diluted in the same serum free culture medium where the cells were put in during treatment. Concentrations were calculated from a dilution series of standards using the Luminex software. Each experiment was performed in triplicate and concentrations of mediators are given as pg/mL. When a mediator is expressed below the detection level, the detection level for that mediator is used as the missing value with the “<” indication.

*Statistical analysis.*

Statistical significances ($p<0.05$) between expression levels of healthy and allergic individuals were determined using unpaired Mann-Whitney rank sum test using GraphPad Prism 4.0 for Windows.
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Results

Baseline and induced expression of mediators in nasal epithelial cells.

In this experiment we confirmed that nasal epithelial cells can produce cytokines and chemokines at low to moderate concentrations at baseline levels (Table 1). Out of a large panel of cytokines and chemokines we could show expression above the detection level for IL-1RA, IL-4, IL-6, IL-8, G-CSF, IL-10, FGF, GM-CSF, IFN-γ, IP-10, and VEGF. Although the expression for these mediators is generally higher in allergic patients than in healthy controls, the expression is clearly highly variable in individual participants. This pattern of expression reflects our previous data, where we saw higher baseline expression of a multitude of genes in allergic compared to healthy individuals.

Exposure to TNF-α or IL-17 induced expression to a different extent for the different mediators. Table 2 shows that most mediators are induced by both TNF-α or IL-17 albeit to a different extent, while a few mediators (IL-
1RA, IL-10, VEGF) are not induced. For G-CSF, GM-CSF, IFN-γ, IP-10 the induction factor was more pronounced in healthy individuals than in allergic individuals. Interestingly, the opposite was true for IL-6 that showed a much stronger inducing capacity in allergic individuals (TNF-α: 3.62 fold and IL-17: 2.37 fold) than in healthy individuals (TNF-α: 2.27 fold and IL-17: 1.41 fold).

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Allergic status</th>
<th>TNF-α</th>
<th>IL-17</th>
<th>TNF-α + IL-17</th>
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Table 2: Induction of cytokine expression. Upon stimulation with TNF-α, IL-17, or combined TNF-α/IL-17, epithelial cells produce cytokines. We calculated the induction by dividing production after stimulation by production in medium control.
Synergistic effect of TNF-α or IL-17 on mediator expression in nasal epithelial cells.

To study a potential synergistic effect of TNF-α and IL-17 it is important to show that TNF-α did not affect IL-17 expression levels and conversely that IL-17 did not affect TNF-α expression. Indeed this was not the case (data not shown). Table 2 shows that the simultaneous addition of TNF-α and IL-17 has a different effect for the various cytokines. For some cytokines (IL-4, IL-9, IL-10, FGF, GMCSF, IP10, and VEGF), the inducing effects of TNF-α and IL-17 seems additive, with the expression level after the simultaneous addition merely being the sum of the expression of each factor individually. For IL-1RA and FGF, the simultaneous addition of TNF-α and IL-17 leads to an expression level that is not different from when either of the factors is added alone. However, what stands out most from the comparison is the synergistic effect between TNF-α and IL-17 on the expression of IL-6, IL-8, GCSF, and IFN-γ. Figure 1 shows a moderate synergistic effect for IL-6, a clear synergistic effect for both IL-8 and GCSF, and a very strong synergistic effect for IFN-γ.

Figure 1. Synergy factors. Synergistic effect of combined TNF-α and IL-17 stimulation compared to stimulation with the factors alone. Values represented are average (± SD) calculated between the 5 individuals.
Interestingly, there is a difference in the synergistic effect between healthy and allergic individuals. For IL-8, GCSF, and IFN-γ the synergistic effect in allergic individuals is smaller than in healthy individuals. For IL-8 the synergistic effect goes down from 4.8 to 2.5, for GCSF from 3.54 to 1.53 and, most remarkably, for IFN-γ the synergistic effect goes down from 49.0 in healthy individuals to just 1.5 in allergic individuals. Only IL-6 seems to behave in an opposite fashion, with a moderate synergistic effect in allergic individuals (1.6) that seems to be absent (1.1) in healthy individuals.

**Discussion**

Our previous data using a microarray approach on primary nasal epithelial cells did not only show that the cells from allergic individuals are different at baseline from epithelial cells of healthy individuals, but also that they respond differently to the same trigger. In this manuscript we further add to this concept and show that allergic epithelium differs in INF-γ response after stimulation with IL-17 and TNF-α.

The synergistic effect of the simultaneous addition of TNF-α and IL-17 has been reported before \(^{13-15}\), but we could only partly replicate these findings. Bronchial epithelial cells and fibroblast show an increased expression of IL-6 and IL-8 in response to the simultaneous addition of TNF-α and IL-17 \(^4\). In our experiments this is also true for IL-8, although the extent of the reported synergistic effect somewhat larger in bronchial epithelial cells (7.6 times) than in nasal epithelial cells (4.8 times). However, for IL-6 we only observed a moderate synergistic effect in allergic epithelium and not in healthy epithelium. The most striking difference between the bronchial and nasal data is that in our experiments IL-17 alone already induces IL-6 and IL-8, whereas IL-17 was not able to induce these genes in bronchial epithelial cells \(^4\). A difference in response for bronchial and nasal epithelial cells to an identical trigger has been observed before with bronchial epithelial cells
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responding strongly to the addition of lipopolysaccharide (LPS), while nasal epithelial cells are unresponsive towards LPS (data not shown). Why these nasal responses would be different from the data in bronchial epithelial cells is not clear. Possibly this is related to the constant exposure to environmental stimuli in the nose, with the bronchial epithelial cells being rather shielded from these stimuli. A similar difference in the response to LPS has also been observed for gut and kidney epithelial cells, with the gut epithelial cells being refractory for LPS and the kidney epithelium responding strongly to LPS \(^{16;17}\). The absence of a clear response to ubiquitous signals (LPS) in the nasal mucosa could perhaps be compensated by a stronger response to inflammatory signals like TNF-α and IL-17.

The use of a broad panel of cytokines in the readout of the synergistic effect of TNF-α and IL-17 allowed us to discover that in addition to IL-6 and IL-8, also GCSF and INF-γ show a synergistic response. The most relevant observation of this work must be that the synergistic effects differ between healthy epithelium and allergic epithelium. Although for most mediators these differences are small, the difference for INF-γ is very large.

As a result, the concentration of INF-γ produced by epithelial cells after the combined exposure to TNF-α and IL-17 is 62-fold higher than after TNF-α exposure and 65-fold higher than after IL-17 exposure (1297 pg/mL versus 20.7 pg/mL, respectively 19.7 pg/mL) in healthy individuals. In allergic individuals we can find a completely different pattern, with the combined stimulation at most 3.9 times higher than either of the stimuli alone. As a consequence, the synergistic effect of combined stimulation is much lower in allergic individuals, despite that the baseline expression of INF-γ is not that different between allergic and healthy individuals.

Given the importance of INF-γ in the Th1 response in general and the antiviral response in particular, this defective INF-γ response in epithelial cells can have important consequences for both the Th1/Th2 balance in the nasal mucosa and/or the effectiveness of an antiviral response. It has
been shown that allergic individuals are more susceptible to viral infections, and that these viral infections are more prolonged \(^{18-23}\). How this process works and what role the epithelial lining in the airway mucosa and the IL-17 producing T-cells may play in this is yet to be exposed.

The modulating capacity of IL-17 on TNF-α-induced gene expression has been described before and has been attributed to both an increase in the transcription rate of down-stream targets by TNF-α and an inhibition of mRNA degradation by IL-17. Although the precise mechanisms still need to be elucidated, mRNA-specific degradation involves defined sequences in the 3' untranslated region of mRNAs that are targeted by small regulatory RNAs called microRNAs or miRNAs \(^{24}\). Over 700 of these 20-25 base pair long miRNAs have been identified up to now and although not all of these miRNAs are thought to be involved in mRNA degradation \(^{25}\), the numbers of these miRNA are sufficiently large to be able to co-regulate a substantial number of genes. In this respect it would be interesting to see if IL-17 affects specific miRNAs and whether these miRNAs target TNF-α-induced mRNAs.

The cellular source of IL-17 in the nasal mucosa is unclear. A likely source could be the recently identified Th-17 cell, a new subtype of T helper lymphocyte for which the exact function is still elusive, but which is uniquely marked by its high level of IL-17 expression \(^{26}\). In chronic bowel disease a marked up-regulation of IL-17 has been reported, indicating IL-17 could function as an enhancer of inflammatory processes \(^{10}\).

In our previous experiments with primary nasal epithelial cells the expression profiling showed that the allergic state of an individual leaves an imprint on the epithelial cells. This imprint itself is not necessarily responsible for the allergic phenotype of patients, but it does result in a different response to allergens in allergic individuals compared to healthy individuals. Now we show that this imprint can also have a direct impact on the Th1/Th2 balance, as in allergic individuals the impaired INF-γ response will affect the ratio between IL-4 (the hallmark cytokine of the Th2 response) and INF-γ (the
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hallmark cytokine of the Th1 response) \(^{27}\). As an additional consequence this deregulation of INF-\(\gamma\) may also be responsible for a reduced antiviral response observed in allergic individuals.

Reference List


