Expression of mRNA for IL-22 Binding Protein in the Bronchoalveolar Fluid after Inhaled Allergen Challenge in Subjects with Asthma

Alexander Behnke1, Franziska Trudzinski1, Quoc Thai Dinh2 and Sebastian Fähndrich3

1Department of Internal Medicine V – Pulmonology, Allergology, Intensive Care Medicine, Saarland University Hospital, Homburg, Germany
2Department of Experimental Pneumology – Saarland University Hospital, Homburg, Germany
3Department of Pneumology/Intensive Care Medicine, University of Rostock, Germany

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Introduction

Asthma is a reversible airway obstruction that is characterized by hyperirritability of the airways, airway inflammation and remodeling of the airways that is associated with constriction of airway smooth muscle, hyper secretion of mucus, edema and thickening of the basement membrane underlying the airway epithelium [1].

Allergen specific T helper (Th) 2 cells play a key role by the activation and chemotactic recruitment of eosinophil granulocytes in the lung tissue, which is mediated especially by Th2 cytokines such as IL-4, IL-5, and IL-13 [2-8]. These cytokines itselfs produce different mediators, that sustain airway inflammation [9,10].

Recent findings provide some evidence that another subset of T helper cells, Th22 cells, which produce IL-22, may play a role in severe asthma [8]. Previous publications reported elevated levels of IL-22 mRNA in peripheral blood mononuclear cells from individuals with atopic that correlate with the severity of asthma [11-13]. IL-22 is a cytokine belonging to the IL-10 family. It binds to a heterodimeric receptor that consists of two chains: IL-22R1 and IL-10R2 chain [14,15]. Lung epithelial cells - but not mononuclear cells - express the IL-22 receptor [14,16]. Effector functions of IL-22 depend on the presence of other cytokines, e.g. IL-17A. Previous studies described inhibitory effects of IL-22 on the antigen-induced eosinophilic inflammation in airways [17]. In a mouse model of Th2-induced lung inflammation, IL-22 was protective and reduced levels of IL-13 and IL-25, which was associated with a decline of eosinophil recruitment [17].

To gain more information about IL-22 biology in asthma, we do not only consider mRNA expression for IL-22, IL-22 receptor, we also investigated mRNA expression for IL-22-binding protein (IL-22BP), a soluble receptor for IL-22. The role of this natural antagonist is still unknown. Others demonstrate that IL-22-induced immune suppression was abolished by IL-22 BP, which was secreted by immature dendritic cells and neutralized IL-22 with higher affinity than IL-22R1 as natural antagonist of IL-22 [14]. However, the role of IL-22 BP in asthma remains to be elucidated. Therefore, we asked for the mRNA expression of IL-22BP in the bronchoalveolar fluid (BALF) of individuals with asthma after segmental allergen challenge. In a second step, we investigated, if expression of mRNA for IL-22BP in peripheral blood mononuclear cells (PBMC) is inducible by co-stimulation with Th2 cytokines IL-4, IL-9, and GM-CSF in a time-dependent manner.

Materials and Methods

Inhaled allergen challenge and determination of the PD20

We performed Inhaled allergen challenge according to Virchow et al. [19] without any changes. All of the nonsmoking allergic patients with asthma gave their written informed consent. The local ethics
committee approved the present study. Allergic asthma was diagnosed by the following criteria: Positive skin prick test result (to rye pollen, birch pollen, grass pollen, cat, or Dermatophagoides pteronissinus/ farinae) and specific IgE. We used the specific allergen for inhaled allergen provocation. Concomitant medication: We stopped corticosteroids 14 days before inhaled/segmental allergen challenge. Patients inhaled 1 mL of normal saline from a jet nebulizer (Pari Boy; Pari Werke, Starnberg, Germany; breathing at tidal volume, average: 25–40 breaths). We used allergen as standardized extracts from Allergopharma (Reinbeck, Germany) (including grass and cat), later from ALK-Abelló (Bornheim, Germany) (10 biological units/mL), and subsequently from HAL Allergy GmbH (Düsseldorf, Germany) (1000 allergen units/mL for rye and birch; 100 allergen units/mL for Dermatophagoides pteronyssinus) (Tables 1 and 2).

Table 1: Individuals with asthma (N=3) that underwent inhaled allergen challenge for the investigation of the BALF.

<table>
<thead>
<tr>
<th>Proband</th>
<th>Sex</th>
<th>Age [Years]</th>
<th>Allergen</th>
<th>FEV1 predicted</th>
<th>% Total-IgE [kU/l]</th>
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<tbody>
<tr>
<td>1</td>
<td>W</td>
<td>24</td>
<td>Animal hair, grass pollen</td>
<td>97</td>
<td>692</td>
</tr>
<tr>
<td>2</td>
<td>W</td>
<td>24</td>
<td>Hay, grass pollen</td>
<td>94.6</td>
<td>211</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>35</td>
<td>Animal hair</td>
<td>56.8</td>
<td>320</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>19</td>
<td>Grass pollen</td>
<td>106</td>
<td>502</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>24</td>
<td>Dust, pollen</td>
<td>99</td>
<td>2003</td>
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<td>6</td>
<td>M</td>
<td>33</td>
<td>Birch</td>
<td>97</td>
<td>264</td>
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<td>24</td>
<td></td>
<td>91.7</td>
<td>665.3</td>
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Table 2: Characteristics of individuals with asthma and healthy controls that donate blood for purification of PMNCs.

<table>
<thead>
<tr>
<th>Proband</th>
<th>Age</th>
<th>Sex</th>
<th>Allergen</th>
<th>FEV1 Predicted %</th>
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<tr>
<td>A 1</td>
<td>23</td>
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<td>Grass pollen</td>
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<td>A 5</td>
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<td>Dust, pollen</td>
<td>99</td>
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<td>A 6</td>
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Segmental allergen challenge

We performed segmental allergen provocation according to Virchow et al. without any changes [19]. Bronchoscopy was done by using an Olympus bronchoscope (Olympus, Hamburg, Germany) via the nasal or oral route. Monitoring occurred by echocardiography and pulse oximetry during and after bronchoscopy. After local anesthesia [19]. 2.5 mL of saline was instilled into the lower left lobe (B8) and the lingular bronchus (B4 or B5 left) as a control. Lavage of the bronchus 8 (right) and the right middle lobe (B4 or B5) was performed with 100 mL of NaCl. Ten × PD20 of allergen diluted. B8 was lavaged 10 minutes after endoscopic allergen deposition. Repetition of bronchoscopy with lavage of segments B4 or B5 in the right and the left lung occurred after 18, 24, 42, 72, or 162 hours. The monitoring was practiced for 2 to 4 hours after bronchoscopy. Patients received supplemental oxygen and β2-agonists if needed [18].

Isolation of PBMCs occurred according to Luttmann et al. [19]. We collected 30 mL of venous blood into plastic syringes containing 0.2% EDTA from healthy subjects and individuals with asthma. All subjects gave their informed consent. PBMCs were separated on a gradient of Biocoll with a density of 1.077 g/L (Biochrom, Berlin, Germany) and resuspended in RPMI supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco), 100 IU/ml penicillin, 100 μg/ml streptomycin (Seromed), 2 mM l-glutamine at a concentration of 1 × 10^6/ml and cultured. Cells were counted in a Neubauer-counting chamber.

Cell cultivation and co-incubation: Cells were cultured on a 24-well-plate (each well with 1 ml) at 37°C (atmosphere of CO2 5%). As
positive controls we used as unspecific stimulation of the cells Phorbol-myristat-Acetat (PMA-in a concentration of $10^{-10}$ M) (Sigma-Aldrich, Munich, Germany) and Calcium-Ionophor (Sigma-Aldrich, Munich, Germany) (concentration $10^{-6}$ M). Co-incubation with the cytokines IL-4 (ImmunoTools, Friesoythe, Germany), IL-9 (ImmunoTools, Friesoythe, Germany), GM-CSF (ImmunoTools, Friesoythe, Germany), and IL-22 (MWG Biotech, Ebersberg, Germany) occurred in various concentrations (10 ng/ml and 100 ng/ml). The duration of the stimulation varied according to the protocol (3 h, 12 h, 24 h, 48 h, 72 h and 96 h). PBMCs were washed with PBS after stimulation/co-incubation with the cytokines. For RNA-isolation, cells were solved in TriZol-Reagenz (Sigma-Aldrich, Munich, Germany) (Lysis-Puffer) and stored at -80°C.

**Isolation of mRNA:** MessengerRNA was prepared, using the Dynal mRNA-Direct-Kit (Dynal-Biotech, Oslo, Norway) according to the recommendations of the manufacturer (Mini-Scale-quality). Cells were solved with binding/lysis puffer. The clean Bead-mRNA-aggregate remained after washing with puffer A and B. Finally, the separation of the beads from the mRNA molecules occurred by rising temperature.

**reverse transcription-PCR (rt-PCR):** The expression of IL-22, IL-22-BP, IL-22-R1 and IL-22-R2 was assessed by rt-PCR (Thermocycler MasterCycler Gradient Eppendorf, Hamburg) in PBMCs from healthy controls and individuals with atopic asthma after co-incubation with PMA, IL-4, IL-9, GM-CSF and IL-22 in a time dependent manner. The same method was used for mRNA expression for IL-22 BP and IL-22 in mononuclear cells of the BALF after segmental allergen provocation. In this study, we used the RevertAid-Kit (MBI, Erlangen). We obtained complementary cDNA by reverse transcription with Random-Hexamer-Primer and Oligo-(dT)-Primer in addition to RNAse inhibitor. The amplification of the relevant cDNA with specific primers to IL-22, IL-22-BP, IL-22-R1 and IL-22-R2 followed. As positive control, mRNA expression for housekeeping-gene β-actin was assessed. The following primers were used: IL-22 sense, 5´-AGC AGC CCT ATA TCA CCA ACC-3´; IL-22-antisense, 5´-GCC TTA TAT GCA GGA GGT GGT-3´; IL-22-BP-sense, 5´-TGG GAG GGC ACT TAC TGG CAA CA-3´; IL-22-BP-antisense, 5´-CTC TGT GAG CCC CCT TAT AAA CC-3´; IL-22-R1-sense, 5´-AGT CTA GCT GTG CCG AGT GAA GA-3´; IL-22-R1-antisense, 5´-AAG CGT AGG GGT TGA AGG T-3´; IL-10-R2-sense, 5´-ACA TTC GGA GTG GCC ACT TAT GCA CCT-3´; IL-10-R2-antisense, 5´-TCT GCA TCT GAG GTC CAA TGT-3´; β-actin-sense, 5´-AGC AGG AAA TTC GTC GTG-3´; β-actin-antisense, 5´-CAG GGT ACA TGG TGC TGC-3´.

**Results**

**Induction of mRNA expression for IL-22 BP and IL-22 in mononuclear cells of the BALF after segmental allergen provocation**

Each of the two probes of mononuclear cells from the BALF of individuals with atopic asthma (n=3) after segmental allergen provocation showed mRNA expression for IL-22 BP and IL-22. In contrast, the mononuclear cells of the BALF from the not provoked control bronchus segments showed mRNA expression for IL-22 but not for IL-22 BP (Figure 1).

**Induction of mRNA expression for IL-22 BP in PBMC after stimulation with IL-4**

We found expression for mRNA of IL-22-BP in PBMCs from individuals with atopic asthma and healthy controls after incubation with IL-4 (after an incubation period of 12 hours, IL-22 BP expression hold on up to 96 hours). Unstimulated PBMCs from individuals with asthma and healthy controls did not show any expression of mRNA for IL-22 BP.

There was no expression for mRNA of IL-22-BP from PBMCs of individuals with atopic asthma and healthy controls neither without stimulation nor after stimulation with PMA, IL-4, IL-9, GM-CSF and IL-22 (incubation period of 12 hours and 96 hours) showed in Figure 2.

We assessed no mRNA expression of IL-22-R1 in PBMCs from individuals with asthma and healthy controls neither without stimulation nor after stimulation with PMA, IL-4, IL-9, GM-CSF and IL-22.

We found a constitutive mRNA-expression of IL-10-R2 in stimulated (PMA, IL-4, IL-9, GM-CSF, and IL-22, incubation period of 12 hours and 96 hours) and unstimulated PBMCs from individuals with asthma as well from healthy controls.

The expression of mRNA for IL-22 was continuously in all probes (stimulated PBMCs with PMA, IL-4, IL-9, GM-CSF, and IL-22 after an incubation period of 12 hours and 96 hours and unstimulated PBMCs from individuals with asthma as well from healthy controls).
Discussion and Conclusion

We found expression for mRNA of IL-22 BP in mononuclear cells of the BALF from individuals with atopic asthma after segmental allergen inhalation challenge in our preliminary study. In vitro, we could induce mRNA expression for IL-22 BP in PBMCs from individuals with atopic asthma and healthy controls after co-incubation with the Th2 cytokine IL-4. In accordance with others, we found continuous mRNA expression for IL-22 – and not for the both receptor chains - in PMCs and mononuclear cells of the BALF [15,20].

Although protein expression for IL-22 BP remains to prove, our preliminary study suggests that IL-22 BP may play a role in a Th2 cytokine driven allergic inflammation as natural antagonist for IL-22.

After allergen exposure, allergen specific T helper (Th) 2 cytokines cause airway inflammation by the recruitment of additional T cells and eosinophil granulocytes in the lung tissue through secretion of a wide range of different pro inflammatory mediators that sustain allergic inflammation [6,8].

Allergic inflammation induced by allergen inhalation is associated with high levels of Th2 cytokines IL-4, IL-5, IL-13 and elevated numbers of eosinophils in the BALF [21]. We do not only found mRNA expression for IL-22 BP in contrast to controls in our in vitro model of a Th2 emphasized allergic inflammation, we also could induce IL-22 BP by stimulation of PBMCs with the Th2 cytokine IL-4 in vitro.

Previous publications reported that the cytokine IL-22 could play a role in asthma. Serum levels of IL-22 are higher in patients with severe asthma than in patients with mild asthma and healthy controls. Moreover, plasma concentrations of IL-22 tended to increase with the severity of the disease [13].

According to observations in mouse models, IL-22 seems to have anti-inflammatory effects with regard to allergic inflammation in airways [17]. IL-22 neutralization during antigen challenge enhanced allergic lung inflammation with increased Th2 cytokines. Consistent with this, recombinant IL-22 given with allergen challenge protects mice from lung inflammation [22].

Our preliminary study put the question, if there is a quantitative relationship between protein expression of IL-22 BP, IL-22 and IL-4 as well as eosinophilia in the BALF of individuals with asthma after allergen inhalation. For this purpose, a quantitative assessment of protein expression for IL-22 BP and a correlation with the quantitative assessment of Th2-cytokines and eosinophils in the BALF after allergen provocation should be a target for further investigations in future.

The association of mRNA expression for IL-22 BP with a Th2 driven inflammation is remarkable, because IL-22 BP is the antagonist for IL-22 [22].

The results of our preliminary mRNA expression study should encourage further investigations because a probable dysregulated action of IL-22 by IL-22 BP may be a target for therapeutic interventions in asthma in future.

References

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