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

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Abstract

Background: T helper (Th)17/22 cells may play a role in allergic asthma. Recent findings showed increased mRNA expression for IL-22 in peripheral blood mononuclear cells (PBMC) from patients with asthma. Thereby, the role of the natural antagonist of IL-22, IL-22 binding protein (IL-22 BP), a soluble receptor for IL-22, remains to be elucidated. In this study, we investigate the expression of mRNA for IL-22 BP in individuals with asthma.

Methods: We assessed the effect of allergen inhalation challenge on mRNA-expression for IL-22 and IL-22 BP, and IL-22 receptor in mononuclear cells of the broncheoalveolar fluid (BALF) from individuals with allergic asthmatic. Furthermore, *in vitro* we investigated mRNA expression for IL-22 and IL-22 BP, and IL 22 receptor after co-stimulation of peripheral blood mononuclear cells (PBMC) from individuals with asthma and normal controls with Th2 cytokines IL-4, IL-9, and GM-CSF in a time-dependent manner.

Results: The expression of mRNA for IL-22-BP in PBMCs from healthy controls and individuals with asthma could be found after co-stimulation with IL-4 after a minimum of 12 hours, which hold on for 96 hours. The expression of mRNA for IL-22 BP in subjects with atopic asthma was discovered in mononuclear cells of the BALF after segmental allergen inhalation challenge. Our findings suggest a possible role for IL-22 BP in allergen-induced airway responses.

Keywords: Asthma; Broncheoalveolar fluid; Allergen challenge

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 itselves 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 heterodimer 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 broncheoalveolar 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.

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).

Proband	Sex	Age [Years]	FEV1 Baseline [L]	FEV1 [% pred]	Total-IgE [kU/l]	specific IgE [kU/ml]	Inhalation-allergen-Test	Diagnosis
1	W	24	3.8	109	52	*D. pteron* 13.8 *D. farinae* 16.9	Positive	Asthma
2	W	24	3.8	110	312	Birke 3.7 *D. pteron* 63.8 *D. farinae* 81.7	Positive	Asthma
3	M	35	6.3	124	116	Birke 35.2 Roggen 3.8	Positive	Asthma

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

Proband	Age	Sex	Allergen	FEV1 % predicted	Total-IgE (kU/l)
A 1	23	F	Animal hair, grass pollen	97	692
A 2	23	M	Hay, grass pollen	94.6	211
A 3	22	F	Animal hair	56.8	320
A 4	19	F	Grass pollen	106	502
A 5	24	M	Dust, pollen	99	2003
A 6	33	M	Birch	97	264
Mean	24			91.7	665.3
K 1	28	M	None	n.d.	n.d.
K 2	19	W	None	n.d.	n.d.
K 3	24	M	None	n.d.	n.d.
K 4	24	M	None	n.d.	n.d.
K 5	26	M	None	n.d.	n.d.
K 6	23	W	None	n.d.	n.d.
Mean	24				

Table 2: Characteristics of individuals with asthma and healthy controls that donate blood for purification of PMNCs.

Patients inhaled a 1:10.000 or 1:1000 dilution of the allergen with subsequent spirometry after 10 minutes. We used the next higher allergen concentration after 10 cumulative breaths. The procedure repeated until observation of a 20% decrease in FEV1. Subsequently, we measured FEV1 at 0.5, 1, 2, 4, 6, and sometimes 8 hours after allergen inhalation. As a criterion for a late-phase reaction we considered a second decline in FEV1 of 15% from baseline (4 to 8 hours after allergen challenge as inclusion criterion). We calculated the individual PD20 by linear interpolation of the relationship between the dose of allergen inhaled and the decline in FEV1 by 20%. For the subsequent segmental allergen challenge we used the 10-fold increased dose of allergen [18].

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 occured 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 CO_2 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) acccording 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 transription-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 CTT CAT AAA CC-3´; IL-22-R1-sense,5`-CTA CGT 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 GGT CAA TGT-3`; IL-10-R2-antisense, 5`- TCT GCA TCT CAG GAG GTC CAA-3`; β-actin-sense, 5`- AGC GGG AAA TCG TGC GTG-3`; β-actin-antisense, 5`- CAG GGT ACA TGG TGG 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).

Figure 1: K=Kontrolle (control – not provoked control bronchus), P=individuals with atopic asthma after segmental allergen provocation.

There was neither mRNA for IL-22 R1 from the BALF of bronchus segments after segmental allergen provocation nor mRNA from the BALF of the control segments.

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 after co-incubation with the cytokines 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).

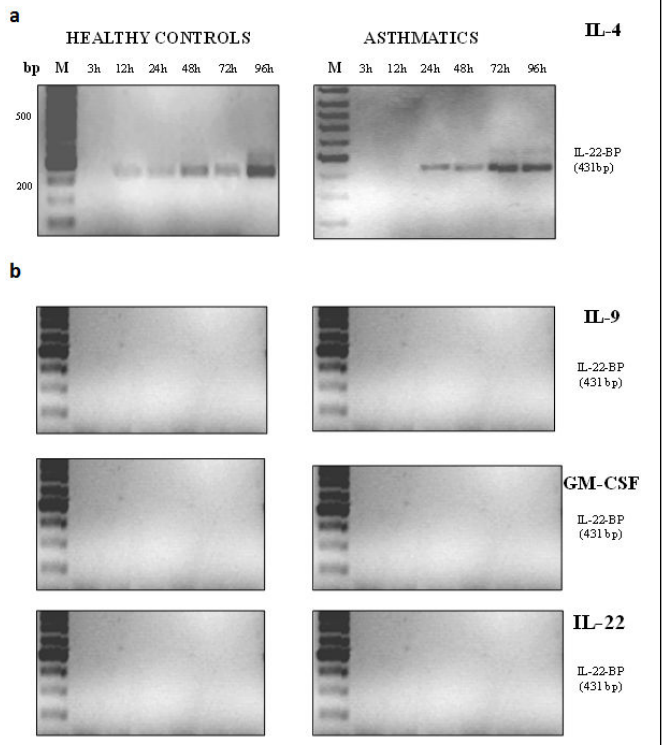

Figure 2: (a) Influence of IL-4 at the expression of IL-22 BP at healthy controls (HC) and individuals with atopic asthma (A), M=100bp-marker. (b) Influence of IL-9, GM-CSF and IL-22 at the expression of IL-22 BP at healthy controls (HC) and individuals with atopic asthma (A), M=100bp-marker.

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 vivo* 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.

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