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Regulation of GATA-3, c-maf and T-bet mRNA Expression in Bronchoalveolar Lavage Cells and Bronchial Biopsies after Segmental Allergen Challenge

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Key Words

Asthma · Atopy · Real-time polymerase chain reaction · Segmental allergen challenge · Transcription factors

Abstract

Background: GATA-3 (GATA binding protein 3) and the proto-oncogene c-maf are Th2-regulating transcription factors that control the expression of interleukin (IL)-4 and IL-5, while T-bet (T-box expressed in T cells) is a Th1-specific transcription factor that controls the expression of interferon (IFN-γ). Allergen provocation in asthmatics induces a Th2-dominated cytokine profile, but so far it is unknown whether the skewed cytokine expression is reflected by the expression of the respective transcriptional regulators. ***Objective:*** The aim of this study was to determine the regulation of Th1- and Th2-specific transcription factors and cytokines in 10 atopic subjects with mild asthma and 5 nonatopic healthy controls at baseline and after segmental sham and allergen challenge. ***Methods:*** The mRNA expression of GATA-3, c-maf and T-bet was determined by real-time polymerase chain reaction in bronchoalveolar lavage (BAL) cells and bronchial biopsies. The percentage of IL-4+, IL-5+ and IFN-γ+ BAL T cells was determined by flow cytometry, and BAL levels of these cytokines were measured by ELISA. ***Results:*** In BAL cells of asthmatics, the mRNA expression of all transcription factors was increased after allergen challenge. In bronchial biopsies, the basal expression of GATA-3 was increased in asthmatics compared to healthy controls but decreased after allergen challenge. Compared to sham challenge, the percentage of IL-5+/CD4+ BAL T cells was increased after allergen challenge in asthmatics while the percentage of IFN-γ+/CD4+ and IFN-γ+/CD8+ T cells was decreased. Expression of c-maf mRNA in BAL cells correlated with IL-4+/CD4+ BAL cells and BAL IL-5 levels. ***Conclusions:*** Segmental allergen challenge in asthmatics leads to increased GATA-3, c-maf and T-bet expression in BAL cells but not in bronchial biopsies.

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Introduction

Allergic asthma is a chronic inflammatory disorder caused by an inappropriate immune response to allergens. The pathophysiology of this response is characterized by the involvement of inflammatory cells which produce a broad range of inflammatory mediators. It has

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Table 1. Clinical characteristics of the study subjects

	Age years	Sex F/M	FEV_1 % of predicted	PC_{20} mg/ml	IgE kU/l
Nonatopic controls					
Median	22	2/3	115	>8	14
Range	18–26		113–123		8–26
Atopic asthmatics					
Median	25	4/6	103.5	3.54	195*
Range	18–33		79–118	0.15–>8	27–502

FEV_1 = Forced expiratory volume in 1 s. * $p < 0.01$ compared to nonatopic controls.

been well established that T-helper type 2 (Th2) lymphocytes and their cytokines play a key role in this inflammatory response [1, 2]. Previous studies have shown that the expression of Th1 and Th2 cytokines is regulated by multiple transcription factors that bind to regulatory elements in the promoters of genes [3, 4]. These protein-DNA interactions result in combinational regulation of genes which leads to specificity and precision of the transcription process [5]. Some of the transcription factors like nuclear factor of activated T cells and activator protein factors are expressed ubiquitously in all T cells, while others are specific for Th1 and Th2 cells. GATA-3 (GATA binding protein 3) and the proto-oncogene c-maf are Th2-specific transcription factors which are expressed in Th2 cells whereas T-bet (T-box expressed in T cells) is a Th1-specific transcription factor which is expressed predominantly in Th1 cells. Accordingly, T-bet regulates the expression of the Th1 cytokine interferon (IFN)-γ [6, 7] and plays a major role in Th1 lineage commitment and IFN-γ production in CD4+ and CD8+ T cells [8]. c-maf seems to regulate predominantly IL-4 expression [9, 10], and GATA-3, besides IL-4, also regulates IL-5 [11, 12] and IL-13 expression [13]. Previous studies have shown that GATA-3 might downregulate IFN-γ expression while increasing IL-4 and IL-5 expression [14–17]. Moreover, GATA-3 might even induce Th2 cytokine expression and chromatin remodeling in committed Th1 cells [18].

Taken together, these analyses of transcriptional regulation of cytokine production show not only a specificity of GATA-3 and c-maf for Th2 cells and a specificity of T-bet for Th1 cells but also the potential of these transcription factors to direct the Th1/Th2 polarization of T cells. Therefore, we assumed that changes in the Th1/Th2 homeostasis in allergic diseases might be reflected by a modified expression profile of the respective transcription factors.

Only few data exist on the local expression of transcription factors in patients with asthma [19–22]. These studies were able to demonstrate alterations in transcription factor expression between patients and healthy controls. We hypothesized that the expression of Th1-/Th2-specific transcription factors might reflect the shift towards a Th2-type cytokine profile and correlates with the respective numbers of cells expressing Th1 and Th2 cytokines during the allergic inflammation. Therefore, we measured the amount of mRNA for transcription factors GATA-3, c-maf and T-bet in bronchoalveolar lavage (BAL) cells and in bronchial biopsies before and after segmental allergen challenge in patients with asthma and in healthy control subjects. In addition, BAL levels of IL-4, IL-5 and IFN-γ were determined by ELISA, and the expression of IL-4, IL-5 and IFN-γ was analyzed in BAL T cells by intracellular cytokine staining. This technique enables to examine the frequency of individual cells within a heterogeneous population that are capable of expressing a particular cytokine upon activation [23].

Patients and Methods

Study Subjects

Ten atopic patients with mild asthma and 5 nonatopic control subjects participated in the study (for patient characteristics, see table 1). All atopics had mild intermittent asthma as defined in the International Consensus Report [24]. They had a history of episodic wheeze with reversible airflow obstruction, and asthma had previously been diagnosed by an independent physician. Each patient had a positive skin prick test, defined as a skin wheal response >4 mm in diameter, to one or more out of twelve common allergens (mixed grass pollen, mixed tree pollen, rye pollen, mugwort pollen, ribwort pollen, *Dermatophagoides pteronyssinus* and *farinae*, cat fur, dog hair, mixed feather, Alternaria and Cladosporia; ALK-Scherax, Hamburg, Germany). The allergen extract which produced the largest wheal response after skin prick testing was used for segmental allergen challenge (mixed grass pollen or *D. pteronys-*

sinus; ALK-Scherax). The chosen concentration was one tenth of the dilution in saline that elicited a 3-mm-diameter skin wheal response. The instilled amount of antigen was 10–1,000 SQ-U diluted in 10 ml of saline solution. Bronchial hyperresponsiveness and the provocative concentration of methacholine that reduced forced expiratory volume in 1 s by 20% (PC_{20}) were determined as described [25]. Patients only used salbutamol when required for relief of symptoms. None were treated with corticosteroids, sodium cromoglycate, theophylline, or leukotriene antagonists. Patients with seasonal symptoms were examined out of the pollen season. The normal controls had no history of allergic or other diseases, negative skin prick tests, normal serum IgE (<100 IU/ml), normal lung function tests and no bronchial hyperresponsiveness (PC_{20} >8 mg/ml). All study subjects were nonsmokers, and no subject had an acute bronchitis 4 weeks prior to the investigations. All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the study following approval by the Ethical Committee of the Hannover Medical School.

Segmental Allergen Challenge

Segmental allergen challenge was performed as previously described [25]. A commercially available endotoxin detection assay (E-Toxate, Limulus amebocyte lysate; Sigma, St. Louis, Mo., USA) was used to demonstrate that the instilled saline and allergen solutions were free of endotoxin. Briefly, all subjects received nebulized salbutamol (1.25 mg), atropine (0.5 mg s.c.) and midazolam (mean 4.8 mg i.v., range 0–10 mg) prior to the bronchoscopy. Lidocaine (maximum: 6 mg/kg) was used to achieve local anesthesia of the upper and lower airways. The bronchoscope (BF 160 P, Olympus Optical, Tokyo, Japan) was wedged into the anterior bronchus of the left lower lobe, and BAL was performed with 6 × 20 ml sterile saline solution. Lavage fluid from the first 20 ml was discarded. A bronchial biopsy was taken from the segmental carina using a radial jaw biopsy forceps (No. 1556; Boston Scientific, Ratingen, Germany). The bronchoscope was then passed into the superior lingular bronchus, and saline solution (10 ml) was instilled as a control challenge. Finally, the instrument was passed to the anterior segment of the right upper lobe, and 10 ml of allergen solution of either grass pollen mix (gp) or *D. pteronyssinus* mix (Dp) were instilled. After 24 h, bronchoscopy was repeated in the subjects following the same premedication, and the superior lingular bronchus and right anterior upper lobe bronchus were lavaged with 100 ml of saline solution followed by segmental biopsy sampling from the allergen-challenged location.

Processing of BAL Cells and Bronchial Biopsies and ELISA for IL-4, IL-5 and IFN-γ

BAL fluid containing cells was filtered through a 100-μm filter and centrifuged, and the supernatant was aliquoted and stored at –80°C until examined by ELISA. ELISA kits for IL-4, IL-5 and IFN-γ were obtained from R&D Systems (Wiesbaden, Germany). The total count of nucleated cells was performed using a Neubauer hemocytometer. Differential cell counts were performed on cytospin slides. Following Pappenheim staining, 300 cells per slide were counted. Cell pellets of 5×10^5 cells and bronchial biopsies were immediately resuspended in 350 μl of RNA isolation buffer (RA1, NucleoSpin RNA II; Macherey-Nagel, Düren, Germany), thoroughly vortexed and snap-frozen in liquid nitrogen.

mRNA Isolation, Reverse Transcription and Real-Time Polymerase Chain Reaction (PCR)

Total cellular RNA was extracted from BAL cells and bronchial biopsies using a commercially available RNA isolation kit (NucleoSpin RNA II; Macherey-Nagel). Reverse transcription was performed with 0.04–0.1 μg of total RNA from each sample in a 40-μl reaction volume for 60 min at 37°C followed by 5 min at 95°C according to the manufacturer's recommendations (Omniscript RT-Kit, Qiagen, Hilden, Germany). Real-time PCR was performed on the LightCycler Instrument using the DNA binding dye SYBR green (LightCycler-Fast Start DNA Master SYBR Green I, Roche Molecular Biochemicals, Mannheim, Germany), as described previously [26]. PCR reaction mix contained 2 μl of PCR master mix supplemented with 2.5 m*M* $MgCl_2$, custom-synthesized primers (MWG Biotech, Ebersberg, Germany) and 2 μl of either external standards, cDNA or untranscribed RNA to exclude DNA contamination, brought to a final volume of 20 μl with water. The specific primers used for PCR were porphobilinogen deaminase (PBGD, M95623, sense: CACACAGCCTACTTTCCAAGC, and antisense: TTCAATGTTGCCACCACACT), GATA-3 (BC006793, sense: AAGGCATCCAGACCAGAAAC, and antisense: GTTAAACGAGCTGTTCTTGGG) [27], c-maf (AF055377, sense: AAATACGAGAAGTTGGTGAGCAG, and antisense: ACACTGGTAACTACACGATGCTG) [28], and T-bet (AF241243, sense: GCGCCAGGAAGTTTCATTT, and antisense: CATTCTGGTAGGCAGTCACG) [29]. After an initial denaturation step at 95°C for 10 min, the PCR reaction was initiated with an annealing temperature of 60°C for 10 s, followed by an extension phase at 72°C for 9 s, and a denaturation cyle at 95°C for 1 s. At the end of each extension phase, fluorescence was observed at 72°C, and the PCR reaction was completed after 45 cycles.

The melting point analysis was carried out by heating the amplicon from 65 to 95°C. Thus, the characteristic melting point was found for each product. DNA contamination was excluded by performing control PCR without reverse transcription. Length and sequence of the product were confirmed by electrophoresis and commercial sequencing (GATC Biotech, Konstanz, Germany).

Preparation of Standards and Semiquantitation of GATA-3, c-maf and T-bet

Purified PCR products were used to create standards for reference (PBGD) and targets (GATA-3, c-maf and T-bet). Amplicons of PBGD and targets were purified with the High Pure PCR Product Purification Kit (Roche Biochemicals). Tenfold serial dilutions were amplified for 45 cycles to create appropriate standard curves. Target and reference genes were analyzed in all the patient samples (at baseline, after saline and after allergen challenge) in duplicate. At the same time, one dilution of the standards, also in duplicate, was analyzed. This was performed in the same LightCycler run. The imported standard curves were then used by the LightCycler software to automatically calculate the amount of target and reference (PBGD) for each sample with reference to the external standard. The target/PBGD ratio was calculated, and the mean ratio of the samples from healthy controls was set to one. Finally, all values were calculated with reference to this mean.

Intracellular Expression of IL-4, IL-5 and IFN-γ in BAL-Derived T Cells

Intracellular cytokine detection was performed after stimulation with anti-CD3/-CD28, as described previously [25]. Briefly,

Table 2. Basic BALF data

BAL	n	Recovery	Total cells	Eosinophils		Neutrophils		Lymphocytes		Macrophages	
		ml	$\times 10^6$	$\times 10^6$	%	$\times 10^6$	%	$\times 10^6$	%	$\times 10^6$	%
Nonatopic controls											
B	5	78	6.0	0.0	0.0	0.2	3.0	0.4	10.0	5.5	87.0
		(73–93)	(3.8–13.0)	(0.0–0.1)	(0.0–1.0)	(0.1–0.3)	(2.0–3.0)	(0.3–1.0)	(5.0–10.0)	(3.3–11.6)	(86.0–91.0)
S	5	82	5.5	0.1	1.0	1.0	16.0	0.5	9.0	4.0	76.0
		(80–94)	(3.8–22.0)	(0.0–0.2)	(0.0–1.0)	(0.2–7.0)	(5.0–32.0)	(0.3–1.8)	(7.0–11.0)	(3.2–13.0)	(59.0–85.0)
A	5	86	8.5	0.1	1.0	2.6	25.0	0.9	8.0	4.9	66.0
		(84–88)	(5.5–38.0)	(0.1–0.4)	(0.0–1.0)	(0.6–25.1)	(10.0–66.0)	(0.5–1.6)	(4.0–11.0)	(4.3–13.2)	(30.0–81.0)
Atopic asthmatics											
B	10	73	4.1	0.0	1.0	0.1	2.5	0.5	11.5	3.6	83.0
		(58–85)	(3.0–15.0)	(0.0–0.1)	(0.0–2.0)	(0.0–0.5)	(1.0–7.0)	(0.2–3.2)	(7.0–21.0)	(2.6–11.4)	(76.0–90.0)
S	10	72	7.6*	0.1	1.0	0.9**	10.0**	0.8	9.5*	5.6	77.5*
		(57–85)	(3.0–58.0)	(0.0–0.6)	(0.0–2.0)	(0.1–33.1)	(2.0–59.0)	(0.3–4.6)	(5.0–14.0)	(2.4–19.7)	(26.0–88.0)
A	10	68*	28.8**	19.5**	64.0**	4.6**	12.5**	2.0*	7.5*	6.1	17.5**
		(34–82)	(6.5–143.0)	(2.1–91.5)	(20.0–82.0)	(0.5–20.0)	(2.0–40.0)	(0.8–18.6)	(4.0–13.0)	(1.7–12.9)	(4.0–44.0)

Fluid recovery, cell counts, and percentage of cells in BAL fluid from nonatopic controls and atopic asthmatics. BAL fluid was obtained at baseline (B) and 24 h after saline (S) and allergen challenge (A). Values are given as medians and ranges. * $p < 0.05$ and ** $p < 0.01$, compared with baseline values.

24-well flat-bottom plates (Nunc, Wiesbaden, Germany) were coated with 1 ml anti-CD3 (OKT3, Orthoclone; Janssen Cilag, Berlin, Germany) at a concentration of 150 ng/ml supplemented with coating buffer containing Na_2CO_3 and $NaHCO_3$ for 2 h at 37°C and 5% CO_2. After washing with RPMI 1640, 1×10^6 BAL cells/ml resuspended in RPMI 1640 supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) were added. Cells were stimulated with anti-CD28 (0.5 mg/ml; Becton-Dickinson, Heidelberg, Germany) in the presence of brefeldin A (1 μM). For intracellular staining, a Cytofix/Cytoperm Kit (BD Pharmingen, San Diego, Calif., USA) was used. After incubation for 6 h at 37°C in a humidified atmosphere of 5% CO_2 in air, cells were washed in staining buffer (PBS containing 1% heat-inactivated FCS and 0.09% sodium azide, pH 7.4–7.6), as recommended by the manufacturer. Anti-CD4 fluorescein isothiocyanate (25 μg/ml; Coulter, Krefeld, Germany) and anti-CD8 peridinin chlorophyll protein (6.25 μg/ml; Becton-Dickinson) were then added, and cells were incubated for 30 min at 4°C in the dark. After a further washing step, cells were fixed and permeabilized with Cytofix/Cytoperm buffer for 20 min at 4°C followed by a washing step with Perm/Wash buffer. For intracellular staining, mouse anti-human IFN-γ, mouse anti-human IL-4 or rat anti-human IL-5, all phycoerythrin conjugated (Becton-Dickinson), were added, and cells were incubated for 30 min at 4°C. As controls, nonspecific fluorescein-isothiocyanate-, phycoerythrin- and peridinin-chlorophyll-protein-labeled antibodies were used (Becton-Dickinson). Flow-cytometric analysis was performed with scatter gates on the lymphocyte fraction using a flow cytometer (Calibur; Becton-Dickinson), as described previously [23]. Positive staining for cytokines was considered when cells were detected above the background level of cells stained with isotype-matched, nonspecific control antibodies in identical concentrations and labeled with the same fluorochrome (phycoerythrin). Cytokine-positive CD4+ or CD8+ T cells were expressed as percentage of total BAL cells.

Statistical Analysis

The Mann-Whitney U test was used for intergroup comparison between atopic asthmatics and nonatopic control subjects. For comparison of paired data within the major study groups (baseline, saline and allergen), the Wilcoxon signed rank test was used for the individual comparisons. The Bonferroni correction for multiple comparisons was used throughout. Probability values of $p < 0.05$ were considered significant. Unless otherwise indicated, all data are expressed as medians and ranges.

Results

BAL Fluid Recovery, Differential Cell Count and T-Cell Subset Distribution

BAL fluid recovery did not differ between baseline, saline-challenged and allergen-challenged segments in nonatopic controls. In atopic asthmatics, recovery was decreased after allergen challenge ($p < 0.05$; table 2). The total cell count was increased after saline and after allergen challenge in atopic asthmatic subjects, whereas no difference was observed in control subjects. After saline challenge, the percentages and absolute numbers of neutrophils were increased in atopic asthmatics as well as in nonatopic controls, but it did not reach statistical significance in the control group. After allergen challenge, the percentages and absolute numbers of eosinophils, neutrophils and lymphocytes were increased in the atopic asthmatics whereas the percentage of macrophages was de-

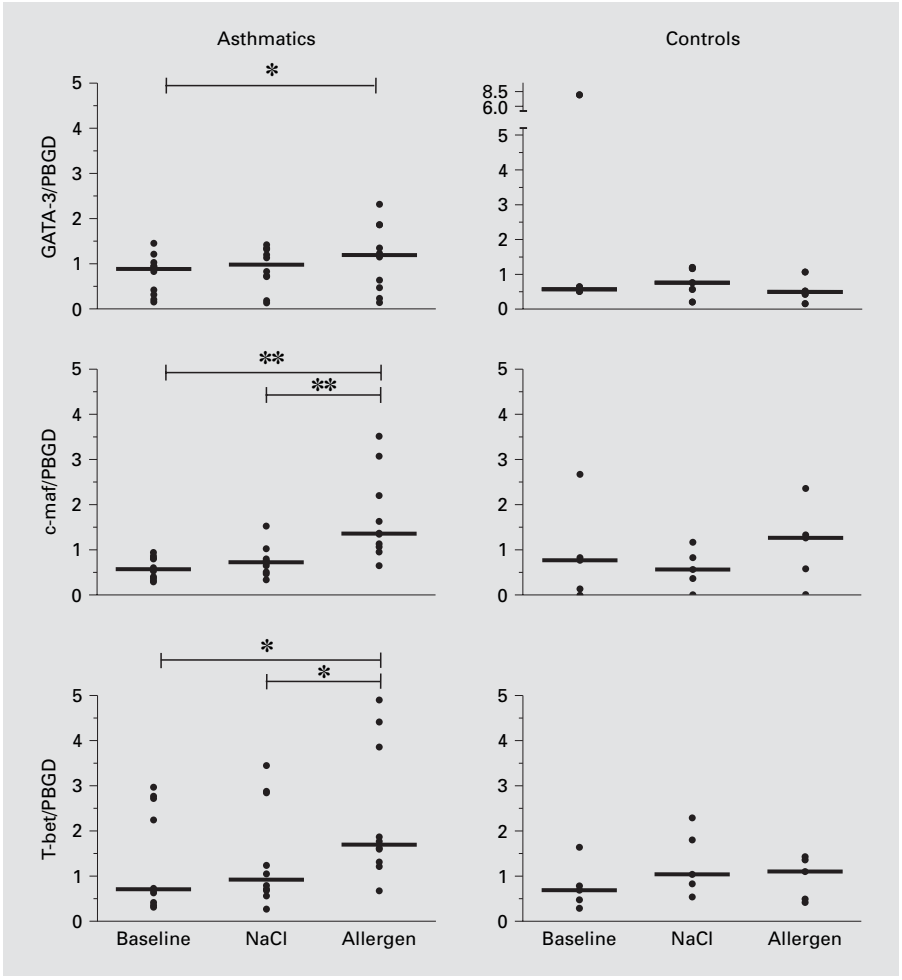

Fig. 1, 2. Quantification of GATA-3, c-maf and T-bet mRNA in BAL cells (**1**) and in bronchial biopsies (**2**) before (baseline) and 24 h after saline (NaCl) and allergen challenge in nonatopic controls and atopic asthmatics. Data are shown as ratio of target mRNA to housekeeping gene (PBGD) mRNA after calculation with reference to an external standard. Bars represent medians. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.05$, vs. nonatopic controls.

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creased. In the nonatopic controls, the percentages and absolute numbers of neutrophils were increased after allergen challenge, but this change did not reach statistical significance (table 2).

The percentage of CD4+ cells significantly increased after allergen challenge in the asthmatic patients while the percentage of CD8+ cells was significantly decreased. However, the absolute numbers of CD4+ and CD8+ cells were increased as a result of increased lymphocyte numbers in BAL. In contrast, no significant changes in percentages and absolute numbers of CD4+ and CD8+ cells were observed for the control group (table 3).

Quantification of GATA-3, c-maf and T-bet mRNA Expression in BAL Cells and Bronchial Biopsies

Quantification of GATA-3, c-maf and T-bet mRNA was performed semiquantitatively with reference to the housekeeping gene PBGD. Appropriate standards produced in triplicate showed stable PCR efficiency for both reference and target genes. In each patient, all samples were then measured in duplicate in one run with a duplicate of a single standard probe. Values were found to be highly reproducible. The results are shown as target/PBGD ratio (fig. 1, 2).

GATA-3, c-maf and T-bet mRNA was constitutively expressed in all BAL cell samples obtained from asthmatic patients and healthy controls, and there was no difference in basal expression between the subject groups. In the healthy control subjects, the expression of GATA-3, c-maf and T-bet in BAL cells was unchanged after saline challenge or after allergen challenge. In contrast, the expression of all three transcription factors in BAL cells was significantly upregulated after allergen challenge but not saline challenge in the asthmatic patients (fig. 1).

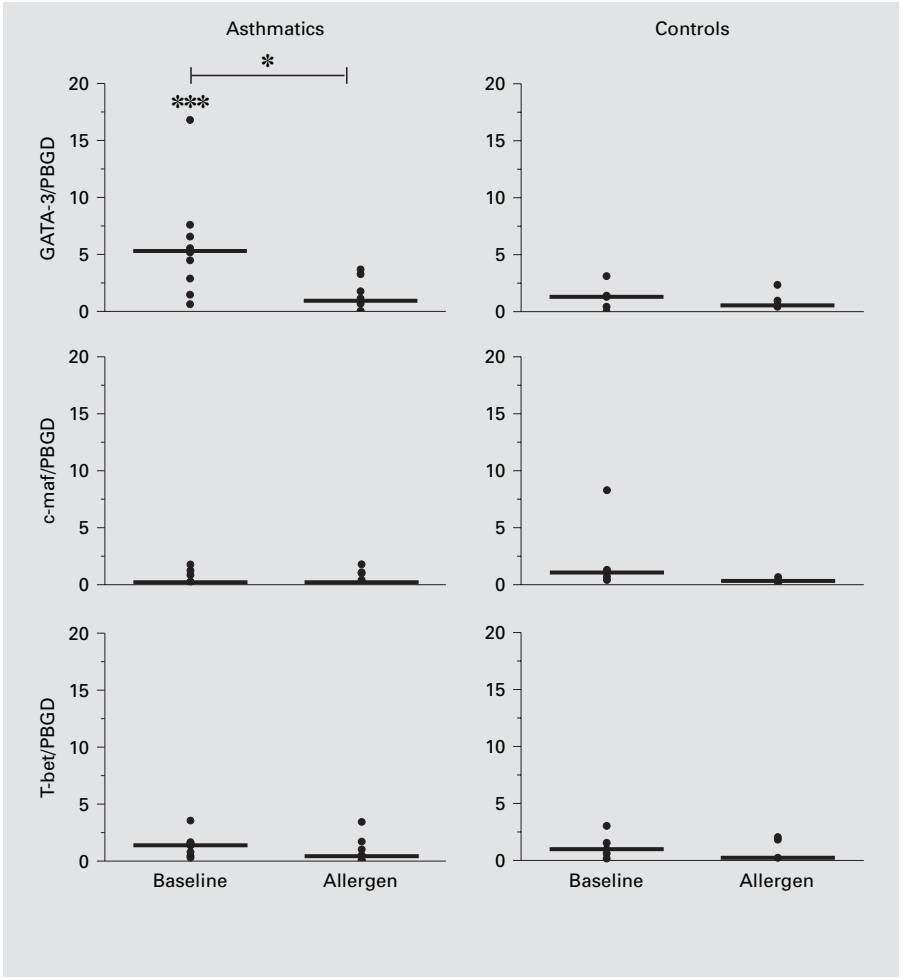

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Table 3. Lymphocyte subpopulations in BAL fluid

BAL	n	CD4+		CD8+	
		$\times 10^3$	%	$\times 10^3$	%
Nonatopic controls					
B	5	178 (80–364)	42.3 (26.5–46.4)	181 (134–500)	42.6 (35.2–60.5)
S	5	224 (129–711)	40.4 (29.2–46.3)	208 (145–733)	41.6 (34.9–45.0)
A	5	516 (114–677)	35.7 (23.4–55.2)	296 (246–717)	44.8 (26.6–60.6)
Atopic asthmatics					
B	10	173 (86–1481)	40.0 (12.0–60.1)	210 (76–1,167)	36.7 (22.6–77.0)
S	10	225 (66–1,777)	39.5 (12.3–59.1)	342 (86–1,393)	36.4 (18.8–76.7)
A	10	840 (264–10,390)**	55.1 (31.3–63.3)*	437 (127–2,347)	13.2 (7.1–46.8)**

Percentages and absolute numbers of lymphocyte subpopulations in BAL fluid from healthy controls and asthmatics. BAL fluid was obtained at baseline (B) and 24 h after saline (S) or allergen challenge (A). Values are given as medians and ranges. * $p < 0.05$ and ** $p < 0.01$, compared with baseline values.

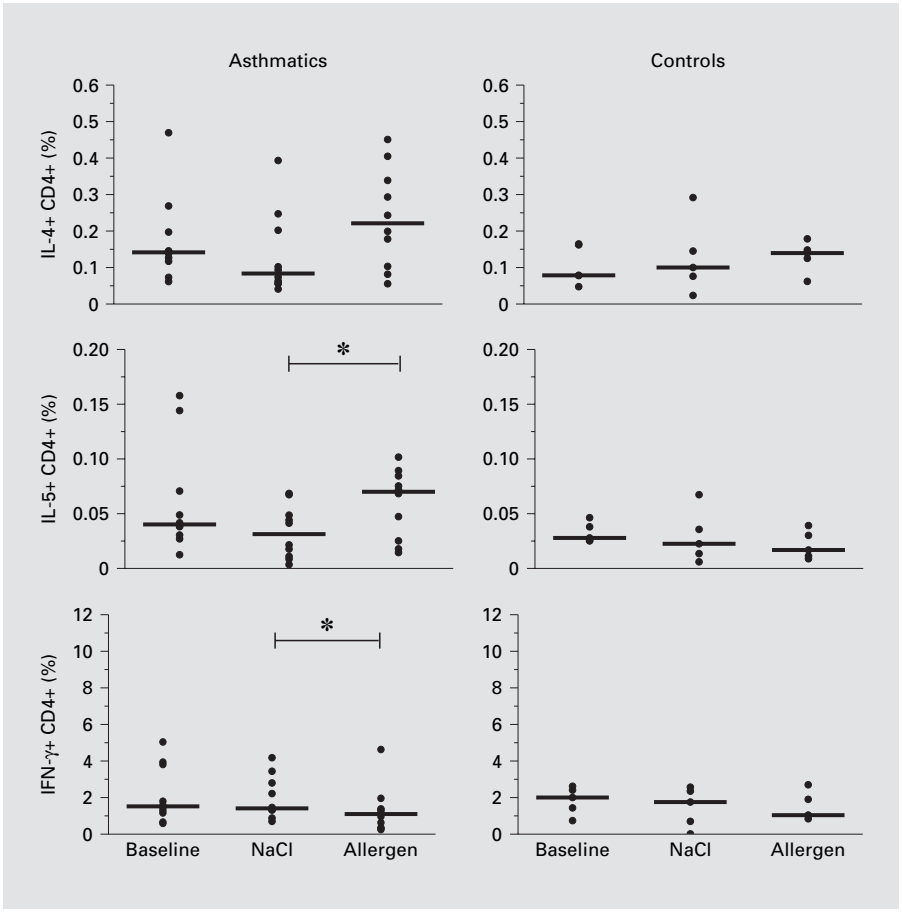

Fig. 3, 4. Percentage of IL-4+, IL-5+ and IFN-γ+ CD4+ (**3**) and CD8+ (**4**) BAL lymphocytes from nonatopic controls and atopic asthmatic patients at baseline, after saline challenge and after allergen challenge. Bars represent medians. * $p < 0.05$, ** $p < 0.01$.

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There was a significant increase in c-maf and T-bet expression after allergen challenge compared to saline challenge while GATA-3 expression was not different between saline and allergen challenge.

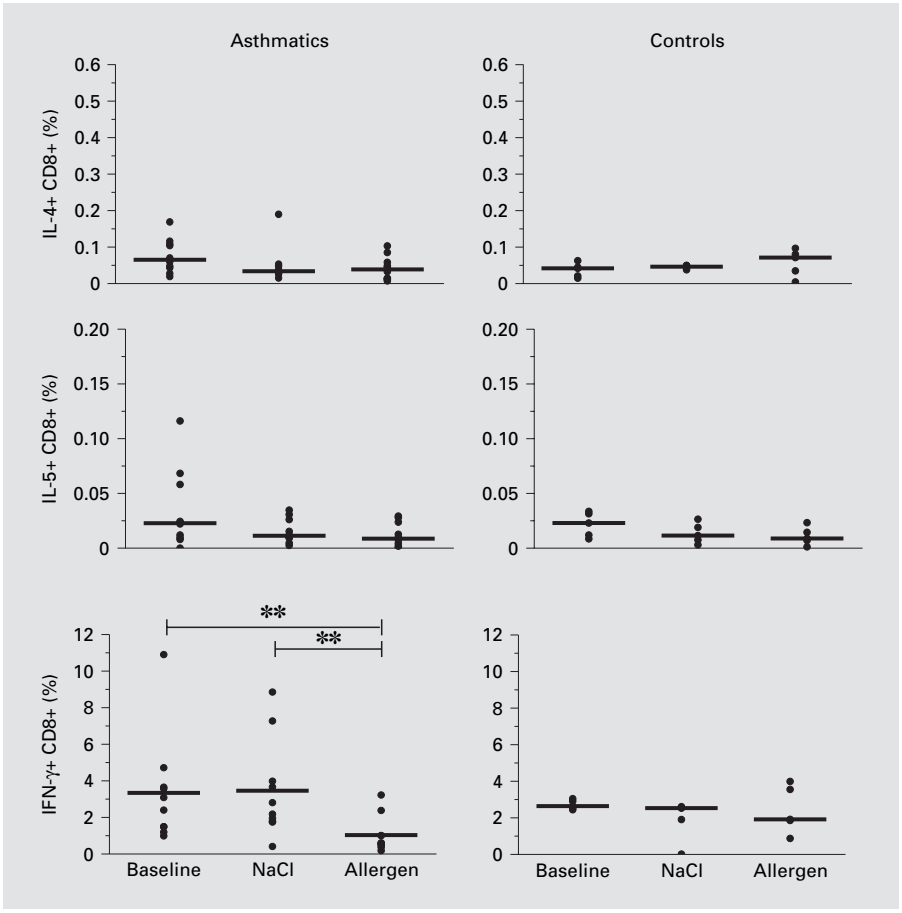
Expression of GATA-3, c-maf and T-bet was found in all biopsy samples of asthmatics and control subjects (fig. 2). Basal expression of GATA-3 mRNA was significantly increased in asthmatic patients compared to healthy controls. Allergen challenge significantly reduced the high GATA-3 expression in biopsies from asthmatic patients, while the expression of GATA-3 in healthy controls remained unchanged. In contrast, expression of c-maf and T-bet was comparable in asthmatics and controls, and it was not influenced by saline or allergen challenge.

IL-4, IL-5 and IFN-γ in CD4+ and CD8+ BAL T Cells

Intracellular cytokines were stained in CD4+ and in CD8+ T cells after stimulation with CD3/CD28 and evaluated by flow cytometry to relate the expression of Th1 and Th2 transcription factors to the proportion of cytokine-expressing T cells. An increased percentage of IL-5+/CD4+ T cells was observed in asthma patients after allergen challenge compared to control challenge, and there was also a tendency towards increased percentages of IL-4+/CD4+ T cells ($p = 0.065$). In contrast, the percentage IFN-γ+/CD4+ T cells was decreased (fig. 3). Regarding CD8+ T cells, a significantly reduced percentage was observed for IFN-γ+ cells after allergen challenge compared to baseline and compared to saline challenge in asthma patients, whereas the percentage of IL-4+/CD8+ and IL-5+/CD8+ T cells remained unchanged (fig. 4). No significant changes occurred in healthy subjects (fig. 3, 4).

IL-4, IL-5 and IFN-γ Protein Levels in BAL Fluid

In healthy controls, IL-5 levels in BAL fluid were low at baseline (34.3, 5.1–43.9 pg/ml), and there was no difference after provocation with either saline (38.4, 6.5–40.4 pg/ml) or allergen (36.8, 7.0–57.4 pg/ml). In patients



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with asthma, IL-5 levels were low at baseline (20.5, 0–29.6 pg/ml) and after saline challenge (18.4, 0–36.3 pg/ml) but increased significantly after allergen challenge (49.9, 0–984.5 pg/ml, $p < 0.01$ compared to baseline and saline challenge).

IFN-γ levels were not significantly different in asthmatics after saline (13.8, 3.0–27.5 pg/ml) or allergen challenge (13.3, 0–28.5 pg/ml) compared to baseline (13.0, 0–31.5 pg/ml), and there was no significant difference in control subjects after saline (14.5, 6.0–72.0 pg/ml) or allergen challenge (30.5, 5.0–68.0 pg/ml) compared to baseline (12.5, 6.5–73.0 pg/ml).

Levels of IL-4 were below the detection limit in all samples analyzed.

Correlation of Transcription Factor mRNA Expression with Cytokine-Producing T Cells and BAL Cytokine Levels

The c-maf mRNA expression in BAL cells from all segments correlated with the respective proportions of IL-4+/CD4+ T cells (fig. 5a) and with the respective IL-5 levels in BAL fluid (fig. 5b). No further correlations were observed for transcription factor mRNA expression in BAL cells and proportions of cytokine-positive T cells or cytokine BAL levels.

Discussion

The aim of the present study was to determine the pulmonary expression of the transcription factors GATA-3, c-maf and T-bet in asthma patients and their regulation in response to segmental allergen challenge. According to the observed shift towards increased Th2 and decreased Th1 cytokine expression after allergen challenge, we hypothesized that the expression of the transcription factors GATA-3, c-maf and T-bet might reflect these changes in the cytokine profile.

Hence we were surprised that the expression of all transcription factors in BAL cells was increased after al-

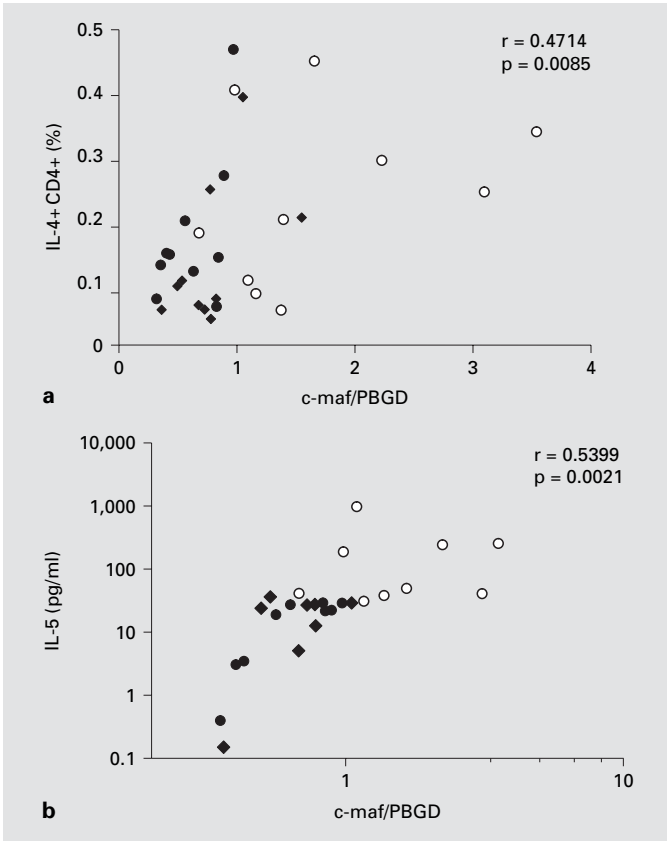

Fig. 5. Correlation of c-maf mRNA expression with IL-4+/CD4+ BAL cells (**a**) and IL-5 levels in BAL fluid (**b**) in asthmatic patients at baseline (●), after saline challenge (◆) and after allergen challenge (○).

lergen challenge. This was unexpected particularly regarding increased expression of T-bet mRNA while numbers of CD4+ and CD8+ T cells producing IFN-γ were decreased. In a previous study, the analysis of human T cells during Th1/Th2 differentiation has revealed that T-bet is upregulated upon activation not only in Th1 cells but also in Th2 cells [30]. Thus, increased T-bet expression after allergen challenge might not only result from activated Th1 but also from activated Th2 cells.

The mRNA expression of T-bet and c-maf in BAL cells was increased after allergen challenge compared to baseline and compared to saline challenge. In contrast, GATA-3 expression was only increased compared to baseline, but there was no difference compared to saline challenge. Thus, the changes in T-bet and c-maf were more pronounced and more specific for the allergic inflammation than the changes in GATA-3.

At baseline, we detected an increased expression of GATA-3 in bronchial biopsies from asthmatic patients compared to healthy control subjects. This is in accordance with previous studies showing increased numbers of GATA-3-positive cells in bronchial biopsies of asthma patients determined at the mRNA level [21] and at the protein level [20]. However, a further study has shown that there was no difference in the GATA-3 protein content in bronchial biopsies from asthmatics compared to healthy controls [31]. These differences might be due to the different techniques used for GATA-3 detection (in situ hybridization vs. immunohistochemistry). Using very sensitive, quantitative, real-time PCR, we were able to demonstrate that GATA-3 mRNA expression was increased in bronchial biopsies from patients with mild asthma. These findings suggest that GATA-3, in contrast to c-maf or T-bet that were not differently expressed between asthmatics and controls, might have a dominant regulatory role in maintaining the disease. Previous studies have shown that GATA-3 is necessary and sufficient for Th2 cytokine gene expression [13, 17]. It directly transactivates the IL-5 gene [11, 12] and is able to downregulate IFN-γ production from developing Th1 cells while inducing IL-4 and IL-5 [15]. Previous studies have established that changes in the Th1/Th2 cytokine profile might be reflected by alterations in the ratio between T-bet and GATA-3 [32, 33]. The interplay of both transcription factors might determine the resulting cytokine expression. Overexpression of T-bet results in Th1 cytokine production [34, 35], whereas the GATA-3 overexpression results in Th2 cytokine production [15, 36]. It is noteworthy that blockade of GATA-3 mRNA expression by antisense oligonucleotides resulted in an abrogation of lung inflammation including inhibition of eosinophil infiltration and Th2 cytokine production and reduction of airway hyperresponsiveness in an ovalbumin mouse model [37]. Thus, these data suggest that the observed increase in GATA-3 expression in bronchial biopsies from asthma patients might indicate a priming for a Th2-type inflammatory response.

Interestingly, increased GATA-3 levels in biopsies were downregulated after allergen challenge to levels comparable with levels of healthy control subjects. This seems to be in conflict with increased IL-5 levels observed in BAL at that time. Since we found upregulation of GATA-3 mRNA expression in BAL cells after allergen challenge, the observed changes in bronchial biopsies might result from a changed cellular composition, e.g. after migration of GATA-3-expressing cells from the bronchial mucosa to the alveolar space. Accordingly, we

observed an increased percentage of IL-5+/CD4+ cells in BAL after allergen challenge.

Like GATA-3, c-maf is involved in Th2 cytokine expression, with a predominance on regulation of IL-4 expression [10, 38, 39]. In addition, a recent study has shown that c-maf regulates IL-5 production in lung CD4+ T cells [40]. This is in good agreement with our observations since mRNA expression of c-maf correlated with the percentage of IL-4+/CD4+ T cells and with IL-5 levels in BAL fluid. Although other studies have shown increased expression of c-maf protein in bronchial biopsies or sputum cells from asthmatics compared to controls [19, 20], we found no difference between asthmatics and controls at baseline. However, c-maf was significantly upregulated in BAL cells after allergen challenge, which might suggest that it plays an important role for the Th2 cytokine production during the allergic inflammation.

We are aware that conclusions from our study are limited because we were not able to show protein levels or activation of the transcription factors. We tried to analyze GATA-3 and activated GATA-3 in bronchial biopsy samples from asthmatics and control subjects by Western blot analysis and using a transcription factor activation assay. In these assays, no GATA-3 or activated GATA-3 was detected (data not shown). Therefore, we conclude that the amount of GATA-3 in the small biopsy samples was too low to be detected by these methods.

Furthermore, the analysis of whole biopsy and whole BAL cell samples impeded the determination of the cellular source of the transcription factors. Previous studies demonstrated that T cells seem to be the major source, but other inflammatory cells, including eosinophils, mast cells or macrophages [19, 21] or even structural cells like epithelial cells, might have contributed to the mRNA levels detected [31].

Taken together, our data demonstrate that changes in Th2 cytokine expression after segmental allergen challenge in asthmatics are reflected by mRNA expression of the respective transcriptional regulators in BAL cells but not in bronchial biopsies. The upregulation of T-bet mRNA in BAL cells after allergen challenge suggests that it is not specific for a Th1-type inflammatory response.

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