

Original article

Decrease of cytotoxic T cells in allergic asthma correlates with total serum immunoglobulin E

Background: Allergic asthma has been linked to an increase in T-helper type 2-like cytokines and T cells, but there is growing evidence for a role of lymphocyte-mediated cytotoxic mechanisms in the pathogenesis of asthma. Therefore, we investigated the cytotoxic potential of different lymphocyte subpopulations in patients with allergic asthma.

Methods: Granzyme A, B, K, and perforin expression in peripheral blood lymphocytes was analyzed using flow cytometry. Soluble granzymes were measured in serum using specific enzyme-linked immunosorbent assays.

Results: Asthmatics had significantly decreased percentages of granzyme and perforin-positive CD4 T cells compared with non-atopic controls. In patients with asthma, the granzyme B and perforin-positive subset of CD8⁺ T cells and natural killer T cells, which represent more differentiated cell populations, were significantly reduced, while this was not observed in the less differentiated granzyme K⁺ subsets. In addition, the serum concentrations of granzyme B were significantly reduced in patients with asthma, while granzyme K concentrations were not different. Interestingly, there was a negative correlation between granzyme A, B and perforin expression in T cell subsets as well as serum granzyme B concentrations and total serum immunoglobulin E. In CD3-negative natural killer cells, no differences in granzyme or perforin expression between patients with asthma and controls were detected.

Conclusion: In allergic asthma, cytotoxic T lymphocyte subsets of a more differentiated phenotype are significantly decreased and this is correlated to serum immunoglobulin E levels.

K. Bratke, F. Haupt, M. Kuepper, B. Bade, S. Faehndrich, W. Luttmann J. C. Virchow Jr

Department of Pneumology, University of Rostock, Rostock, Germany

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Prof. Dr J. Christian Virchow
Department of Pneumology
University Medical Clinic Rostock
Ernst-Heydemann-Str. 6
18057 Rostock
Germany

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Allergic asthma is associated with a characteristic airway inflammation, airway hyperresponsiveness and a variable degree of airflow obstruction (1). However, the underlying etiologic mechanisms are still elusive. According to current opinion, atopic asthma is linked to an increase in T-helper type 2 (Th2)-like cytokines and a decrease in T helper type 1 (Th1)-like cytokines. However, recent studies provide evidence that cytotoxic lymphocytes, such as CD8 T cells and natural killer (NK) cells, as well as lymphocyte-mediated cytotoxic mechanisms, might also participate in the etiopathology of asthmatic inflammation (2–5).

The key molecules of lymphocyte-mediated cytotoxicity are a panel of granule serine proteases, termed granzymes and the pore-forming protein perforin. In humans, five granzymes (Gr) with differing substrate specificity have been identified (6, 7). In the presence of perforin, granzymes are capable of inducing apoptosis in target cells, such as transformed cells and virus-infected cells (8) by activating different cell death pathways (9, 10).

In lymphocytes, granzyme and perforin expression is restricted to NK cells, NK T cells, CD8 T cells, T cell receptor (TCR) $\gamma\delta$ cells, and to a small subset of CD4 T cells (11, 12). During the differentiation of CD8 T cells and NK T cells in peripheral blood, granzyme expression changes from GrA⁺/GrB⁺/GrK⁺ via a small intermediate GrA⁺/GrB⁺/GrK⁺ population to GrA⁺/GrB⁺/GrK⁺ (11). It was further shown that GrK expression is highly restricted to cytotoxic T lymphocytes with a memory phenotype and none to low perforin expression, while GrB expression was only observed in T lymphocytes exhibiting a memory/effector or effector phenotype with high perforin expression (11).

Because it has been reported that interleukin (IL)-4 promotes the development of a CD8 T-lymphocyte phenotype with low perforin and granzyme expression (13), and as elevated concentrations of IL-4 have been associated with acute asthma exacerbations (14–16), we hypothesized that the expression of cytotoxic proteins in T lymphocytes of patients with allergic asthma would be

reduced compared with healthy controls. To substantiate our hypothesis, we analyzed the expression of granzymes A, B, K, and perforin in different lymphocyte subsets (CD4 T cells, CD8 T cells, NK T cells, TCR $\gamma\delta$ cells, NK cells) in patients with allergic asthma and healthy controls using flow cytometry. In addition, soluble granzymes A, B, and K were measured in human serum by specific enzyme-linked immunosorbent assays (ELISA).

Methods

Subjects

Sixteen asymptomatic patients diagnosed as suffering from allergic asthma as well as 17 non-atopic sex- and age-matched controls participated in this study (asthmatics: 10 females, six males, 25 $\pm$ 5 years; controls: 11 females, six males, 26 $\pm$ 4 years). All patients and controls were non-smokers and showed no clinical signs of infection, at least 6 weeks prior to the study. Atopy was confirmed by the UniCAP sx1-test (Pharmacia Diagnostics, Freiburg, Germany) against common inhaled allergens, while all non-atopic controls showed a negative signal in this test. Median serum IgE levels were 27.0 kU/l (range 3.7–122.0) for controls and 212.0 kU/l (range 18.0–2795.0) in patients with asthma. Further patient characteristics are shown in Table 1. All patients gave their written informed consent and the study protocol was approved by the local ethics committee.

Monoclonal antibodies

Anti-human CD4-phycoerythrine (PE) (clone MT310), CD8-PE (clone DK25), CD16-PE (clone DJ130C), CD56-PE (clone C5.9)

and CD3-PE-Cy5 (clone UCHT1) were purchased from Dako (Hamburg, Germany). Anti-human TCR $\gamma\delta$ -PE (clone 11F2) was from BD Biosciences (Heidelberg, Germany). The following antibodies were from ImmunoTools (Friesoythe, Germany): anti-human GrA-fluoresceinisothiocyanat (FITC) (clone GrA-11), GrB-FITC (clone GB11), GrK-FITC (clone GM24C3), perforin-FITC (clone δ G9) as well as FITC-labeled isotype control antibodies.

Intracellular detection of cytotoxic proteins

Ethylenediaminetetraacetic acid (EDTA) anticoagulated–blood was collected by venepuncture. Erythrocytes were lysed by adding 10 ml of FACS lysing solution (BD Biosciences, Heidelberg, Germany) to 2 ml of EDTA–blood for 10 min. After centrifugation (300 g, 5 min), leukocytes were washed with 10 ml of phosphate-buffered saline (PBS; Invitrogen, Karlsruhe, Germany) supplemented with 2% fetal calf serum (Invitrogen). Then, leukocytes were fixed in paraformaldehyde (2% in PBS) for 10 min, washed twice with PBS, and were permeabilized with saponin (0.1% in PBS) for 10 min on ice. Subsequently, leukocytes were incubated with fluorochrome-labeled antibodies against intracellular and extracellular proteins for 20 min, washed twice with saponin (0.1% in PBS), and then analyzed by flow cytometry (FACSCalibur, BD Biosciences, Heidelberg, Germany). The gating strategy to identify lymphocyte subsets and granzyme⁺ as well as perforin⁺ cells is explained in Fig. 1.

Granzyme and IL-4 ELISAs

Granzyme A, B and IL-4 ELISAs (ImmunoTools, Friesoythe, Germany) were performed as described by the manufacturer. Human granzyme K ELISA was performed as previously described (17).

Table 1. Patient characteristics

Sex	Age (years)	Duration of asthma (years)	Total IgE (kU/l)	Specific IgE (CAP class)	FEV ₁ baseline (l)	FEV ₁ (% pred)	Medication
m	37	10	539	Birch (6), hazel (6)	4.8	104	OA
f	25	11	175	<i>D. pter.</i> (5), <i>D. farinae</i> (5)	3.3	95	β
f	22	15	40	Rye (3)	3.3	98	–
m	24	16	281	nd	4.2	93	–
f	23	17	912	nd	2.3	68	β , IC
f	23	2	55	<i>D. pter.</i> (3), <i>D. farinae</i> (3)	4.2	109	CR, β , OA
f	22	10	1130	Rye (3), <i>D. pter.</i> (3)	3.4	nd	β , OC
f	25	13	121	Birch (4), hazel (3)	3.6	101	β , OA
f	21	9	2795	nd	2.8	85	–
m	22	17	358	Rye (6), birch (3)	4.6	nd	–
f	23	6	770	<i>D. pter.</i> (5), <i>D. farinae</i> (5)	3.8	110	CR
f	24	3	18	Birch (3)	3.9	123	IC, β
m	24	22	249	nd	3.3	80	CR, IC, β
f	34	5	39	nd	4.0	116	–
m	19	6	125	nd	4.4	96	–
m	33	24	121	Birch (4), rye (3)	5.7	124	IC, β
Mean	25	12	212*		3.9	100	
SD	5	7			0.8	16	

β , β_2 -agonists; CR, cromoglycate; *D. pter.*, *Dermatophagoides pteronyssinus*; FEV₁, forced expiratory volume in 1 s; IC, inhaled corticosteroids; nd, not determined; OA, oral antihistamines; OC, oral corticosteroids (discontinued 2 months prior to study); SD, standard deviation.

All medications except β_2 -agonists were withdrawn ≥ 7 days prior to the study.

*Median value.

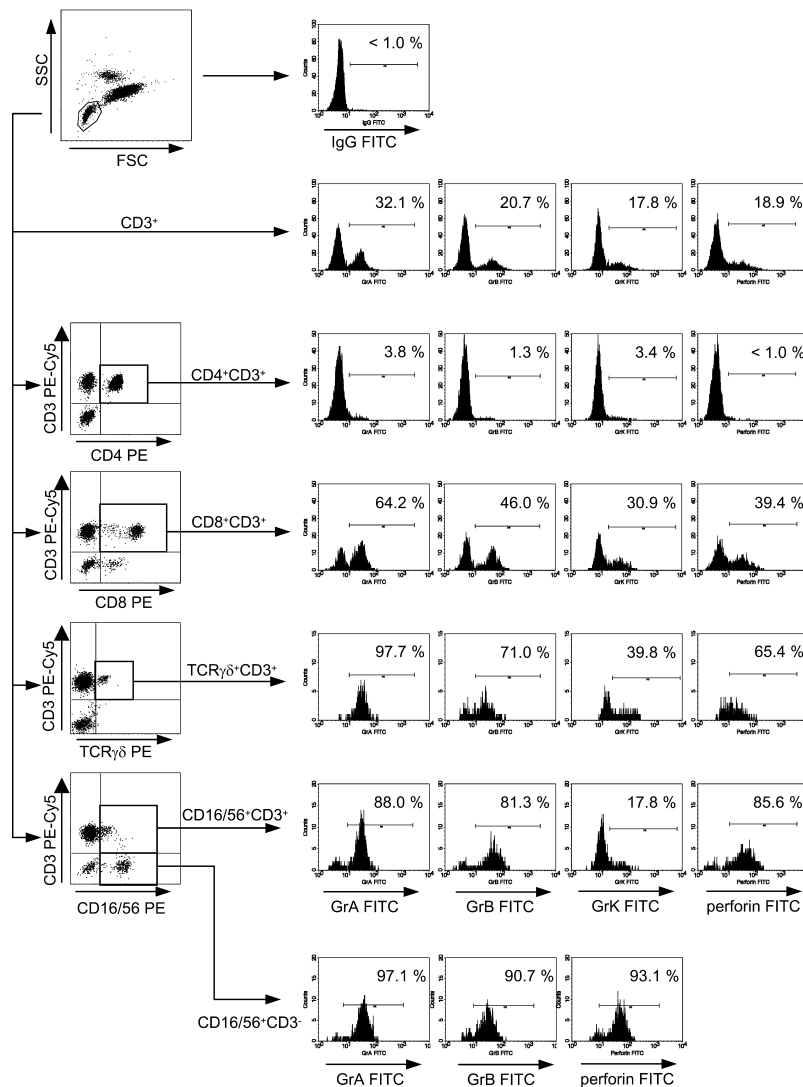


Figure 1. Gating strategy for the identification of granzyme and perforin expressing lymphocytes. T helper cells (CD4⁺CD3⁺), CD8 T cells (CD8⁺CD3⁺), NK T cells (CD16/56⁺CD3⁺), TCRγδ cells (TCRγδ⁺CD3⁺) and NK cells (CD16/56⁺CD3⁻) were identified by dot-plots. The percentage of granzyme- and perforin-expressing cells in lymphocyte subsets was determined in histogram plots. In a histogram displaying all CD3⁺ lymphocytes, granzyme- or perforin-positive cells were defined setting a marker. Subsequently, this marker was copied into the histogram showing the respective lymphocyte subpopulation.

Immunglobulin E determination

The Pharmacia UniCAP 100 system (Pharmacia Diagnostics) was used to determine total serum immunglobulin (Ig)E as well as allergen-specific IgE concentrations. The sx1 screening test for serum IgE against a panel of common aeroallergens was performed using the same system. All measurements were carried out as described by the manufacturer.

Statistical analysis

Data were analyzed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). For the comparison of cohorts, the Mann-Whitney *U*-test was used, except for the analysis of lymphocyte subpopulations (mean ± standard deviation, *t*-test). Correlations were analyzed

using the Spearman rank-sum correlation coefficient. Probability values of <0.05 were used as the generally accepted level of statistical significance.

Results

Granzyme expression in lymphocyte subsets

Flow-cytometric analysis revealed no statistically significant differences in percentages of CD4 T cells (asthmatics: 42.4 ± 6.9%; controls: 44.5 ± 7.2%), CD8 T cells (asthmatics: 24.0 ± 4.5%; controls: 26.7 ± 5.2%), NK T cells (asthmatics: 6.9 ± 3.4%; controls: 8.1 ± 5.6%), TCRγδ cells (asthmatics: 5.1 ± 4.4%; controls:

$3.0 \pm 1.8\%$), and NK cells (asthmatics: $16.0 \pm 6.0\%$; controls: $12.8 \pm 8.3\%$) between patients with asthma and healthy controls.

Intracellular protein staining, however, revealed significantly lower percentages of CD4⁺ T lymphocytes expressing granzyme A, granzyme B, granzyme K, and perforin compared with control subjects (Fig. 2). Among CD8⁺ T lymphocytes, there was a significantly lower percentage of granzyme A, granzyme B, and perforin-expressing cells in patients with asthma, while the percentages of granzyme K-positive CD8 T cells were not different between asthmatics and controls (Fig. 2). NK T cells in asthmatics showed a significant reduction in granzyme B-positive and perforin-positive cells, and a trend towards significantly reduced granzyme A-positive

cells compared with healthy controls ($P = 0.056$), while there were no differences in the percentage of granzyme K-expressing cells compared with controls (Fig. 2). Analysis of lymphocyte subpopulations, which mediate innate immune responses, revealed a significantly reduced percentage of perforin-expressing TCR $\gamma\delta$ cells in patients with asthma, while the percentages of TCR $\gamma\delta$ cells which expressed granzyme A, granzyme B, and granzyme K showed no significant differences between asthmatics and healthy controls (Fig. 2). In NK cells, there were no differences in the expression of granzyme A (asthmatics: 95.5%, range 86.3–99.1%; controls: 95.4%, range: 84.3–98.3%), granzyme B (asthmatics: 90.4%, range 74.5–95.7%; controls: 91.2%, range 71.5–98.0%) and perforin (asthmatics: 90.7%, range 73.6–98.2%; controls: 92.9%,

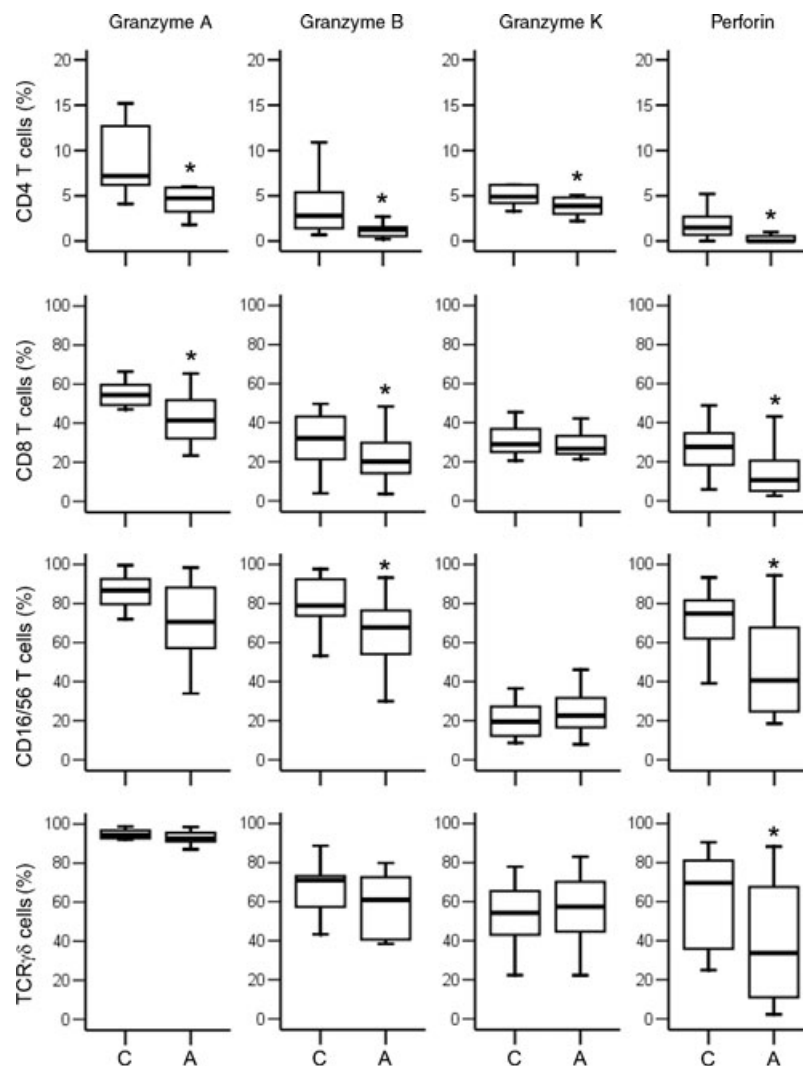


Figure 2. Granzyme and perforin expression in T cell subsets. Percentages of granzyme A, B, K and perforin-expressing CD4 T cells, CD8 T cells, NK T cells, and TCR $\gamma\delta$ cells were determined by flow cytometry as described in methods and Fig. 1. Boxplots display the median (line within the box), interquartile range (edges of the box) and the range of values less distant than 1.5 interquartile ranges from the upper or lower quartile (vertical lines). Asterisks mark significant differences ($P < 0.05$) between asthmatics (A) and controls (C).

range 79.9–99.5%) between patients with asthma and controls. Granzyme K expression in NK cells was not determined, because the low granzyme K expression in CD56^{dim} NK cells allows no clear discrimination between positive and negative cells (11).

Serum concentrations of granzymes and IL-4

Soluble granzymes A, B, and K were determined by specific ELISAs in sera of asthmatics and healthy controls. Patients with asthma showed a significant reduction of soluble granzyme B, while granzyme A and K levels were not different between patients with asthma and controls (Fig. 3). Serum concentrations of IL-4 were below the detection limit of the ELISA assay in most patients and healthy controls (data not shown).

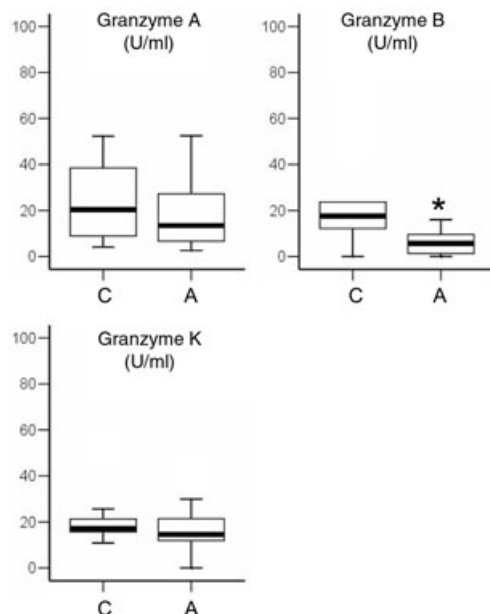


Figure 3. Serum concentrations of soluble granzymes. Soluble granzymes A, B, and K in human sera were determined using specific ELISAs. Boxplots display the median (line within the box), interquartile range (edges of the box) and the range of values less distant than 1.5 interquartile ranges from the upper or lower quartile (vertical lines). Asterisks (*) mark significant differences ($P < 0.05$) between asthmatics (A) and controls (C).

Correlations of cellular and serum granzyme levels with total serum IgE

Since IgE production has been shown to be IL-4-dependent, we correlated total serum IgE concentrations with granzyme expression in lymphocyte subsets and soluble granzyme concentrations. Interestingly, there was a significant negative correlation between granzyme A, B and perforin expression in T lymphocyte subsets and total serum IgE levels (Table 2). This negative correlation was highest in CD4 and CD8 T cells and lowest in TCR $\gamma\delta$ cells (Table 2). In addition, granzyme K expression in CD4 T cells correlated negatively with total serum IgE (Table 2). Furthermore, there was a significant negative correlation of serum granzyme B concentrations with total serum IgE ($r_s = -0.599$; $P = 0.001$), but no correlation between soluble granzymes A and K with serum IgE was detected. No correlation between the expression of cytotoxic proteins in NK cells and total serum IgE levels was found (Table 2).

Discussion

In the present study, we investigated the expression of cytotoxic proteins in different lymphocyte subpopulations in the peripheral blood of patients with allergic asthma and healthy controls using intracellular granzyme and perforin staining as well as specific ELISAs to detect soluble granzymes. This is the first study to show a decrease in differentiated granzyme B and perforin expressing CD8 T lymphocytes and NK T cells in patients with allergic asthma. This was paralleled by a reduced concentration of granzyme B, but not granzyme K, in serum of patients with asthma.

Our results are in agreement with previous reports which described a reduced perforin expression in lymphocyte subsets of patients suffering from other atopic diseases such as atopic dermatitis and allergic rhinoconjunctivitis (18, 19), suggesting that this phenomenon is due to atopic status and not limited to asthma. It differs, however, from a previous study by Arnold et al. which described an increase in perforin-positive lymphocytes in patients with allergic asthma (20). The discrepancy between these studies might be explained by differences in the population studied, as in the study by Arnold et al.

Table 2. Correlation [r_s (P)] of cytotoxic protein expression in lymphocyte subsets with total serum IgE levels

	CD4 T cells	CD8 T cells	CD16/56 T cells	TCR $\gamma\delta$ cells	NK cells
Granzyme A	-0.671 (0.001)*	-0.622 (0.001)*	-0.447 (0.010)*	-0.386 (0.047)*	0.076 (0.678)
Granzyme B	-0.620 (0.001)*	-0.542 (0.001)*	-0.454 (0.009)*	-0.427 (0.026)*	0.006 (0.972)
Granzyme K	-0.483 (0.004)*	-0.341 (0.052)	0.286 (0.113)	0.254 (0.201)	nd
Perforin	-0.486 (0.006)*	-0.356 (0.042)*	-0.449 (0.010)*	-0.279 (0.167)	-0.184 (0.313)

Correlations were analyzed using the Spearman rank-sum correlation coefficient (r_s).

Data were obtained analyzing the whole study population (asthmatics and controls). nd, not determined.

* $P < 0.05$ was regarded as significant.

(20), subjects had relatively low total IgE levels (<120 kU/l in the majority of cases) and the IgE concentrations of healthy controls were not determined. This might be of importance, as in our study we found a significant negative correlation between the cytotoxic protein expression and total serum IgE, suggesting that lower IgE concentrations might be associated with higher perforin expression as reported by Arnold et al. (20). With respect to CD8 and CD56 positive lymphocytes T cells and NK cell subsets were not separated in a previous study (20). Our results show a tendency for a higher percentage of NK cells (which were almost all positive for perforin) and a lower percentage of the respective T cell subsets (which vary in their percentage of perforin-positive cells) in the peripheral blood of patients with asthma. Our findings thus emphasize the importance to distinguish between NK cells and T cells, when CD8- and CD56-positive lymphocytes are being investigated.

Accordingly, our observations provide indirect evidence for the speculation that the observed relationship between cytotoxic protein expression in T lymphocytes and serum IgE concentrations could be mediated by IL-4, which has been shown to regulate IgE synthesis as well as granzyme and perforin expression: IL-4 induces the IgE isotype switch to B cells (21) and is important for the upregulation of both antigen-specific and total IgE (22). Additionally, it has been shown that IL-4 can induce a CD8 T-cell phenotype with reduced granzyme and perforin expression (13, 23). Interestingly, IL-4 serum concentrations were below the detection limit in the majority of patients, suggesting that IL-4 effects rather than the mere IL-4 concentrations are related to the above-mentioned association. It is well established that IL-4 regulates T cell development at the timepoint of the first antigen encounter (24). Furthermore, in a culture system, its influence on CD8 T cell development to a phenotype with low granzyme expression is limited to the first 5 days after activation (23). Therefore, the actual IL-4 concentration is unlikely to reflect the actual repertoire of long-living cytotoxic memory T cells in asymptomatic asthma patients. Instead, it might be more meaningful to compare presumed effects of IL-4 on T lymphocytes (reduced cytotoxic protein expression) with established actions on other lymphocytes like B cells (increased IgE synthesis). Keeping this in mind, it is probable that the

present findings of reduced granzyme/perforin expression in peripheral blood T lymphocytes are caused by asthma/atopy rather than they would have a key role in the disease pathogenesis.

In a previous study, we identified two main populations of cytotoxic CD8 T lymphocytes using granzymes A, B, and K as marker proteins: 1. GrA⁺/GrB⁻/GrK⁺, 2. GrA⁺/GrB⁺/GrK⁻ (11). Interestingly, only the granzyme B-positive CD8 T cell subset is reduced in allergic asthma. This subset consists of highly differentiated memory/effector and effector cells (11) which can be induced by different latent viruses such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), and others (25, 26). Although no conclusive correlations have been reported between CMV or EBV infection in childhood and the onset of atopic diseases (27), it could be speculated that atopic patients with latent virus infections might have acquired an impaired control of such infections compared to non-atopic individuals. This phenomenon has already been described for patients with atopic dermatitis suffering from CMV infection (28). Furthermore, higher levels of serum antibodies against EBV have been detected in patients with atopic dermatitis compared with non-atopic controls (29). But to date, it is not clear if the reduced granzyme/perforin expression in cytotoxic T lymphocytes from atopic individuals is responsible for this phenomenon. Nevertheless, based on previous findings and our own, this hypothesis requires further investigation.

In conclusion, we report a reduced granzyme and perforin expression in different lymphocyte subsets in patients with allergic asthma. This phenomenon was most pronounced in differentiated cytotoxic effector lymphocytes mediating adaptive immune responses (CD4 T cells, CD8 T cells) but less in lymphocyte subsets involved in innate immunity (NK cells, TCR $\gamma\delta$ cells). Furthermore, the expression of cytotoxic proteins granzyme A, granzyme B, and perforin in T cells correlated negatively with total serum IgE, suggesting a link between IL-4-induced IgE synthesis and granzyme and/or perforin synthesis.

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