

Cytokine Profile of Bronchoalveolar Lavage–Derived CD4⁺, CD8⁺, and $\gamma\delta$ T Cells in People with Asthma after Segmental Allergen Challenge

Norbert Krug, Veit J. Erpenbeck, Kerstin Balke, Jan Petschallies, Thomas Tschernig, Jens M. Hohlfeld, and Helmut Fabel

Department of Respiratory Medicine and Applied and Functional Anatomy, Hannover Medical School, Hannover, Germany

T cell–derived cytokines play an important role in the pathogenesis of allergic asthma, but little is known about the cytokine profile of their different subsets. The aim of the present study was to investigate the cytokine production potential of CD4⁺, CD8⁺, or $\gamma\delta$ ⁺ T cells derived from the bronchoalveolar space of mild atopic asthmatic subjects ($n = 11$) and non-atopic control subjects ($n = 9$) before and 24 h after segmental allergen challenge. The cytokine production was determined using the technique of intracellular cytokine detection by flow cytometry. Comparing asthmatic with control subjects we found no difference in the percentage of CD4⁺, CD8⁺, or $\gamma\delta$ T cells in the bronchoalveolar lavage fluid before and after allergen challenge. Before allergen challenge the proportion of cells producing the cytokines interferon (IFN)- γ , interleukin (IL)-2, IL-4, IL-5, and IL-13 was not different in CD4⁺ and CD8⁺ cells. The major difference between the groups was an increased percentage of positive-staining cells for the T helper-(Th)2-cytokines IL-5 and IL-13 in the $\gamma\delta$ T-cell subset. After allergen challenge, all T-cell subsets revealed a decreased proportion of cells producing the Th1-type cytokines IFN- γ and IL-2. The percentage of IL-4- and IL-5-positive cells did not change in all subsets, and there was a decreased proportion of IL-13-positive cells in the CD4⁺ subset. These findings indicate an increased Th2-cytokine profile in $\gamma\delta$ T cells. After allergen challenge, the dysbalance between Th1 and Th2 cytokines was further accentuated by a reduction in Th1 cytokine-producing T cells.

Asthma is characterized as a chronic inflammatory disease of the bronchial mucosa, and T cells have been suggested to play a key role in orchestrating the disease process through the release of cytokines (1). Increased numbers of CD4⁺ T cells have been found in asthmatic airways which show signs of activation (2) and depletion of CD4⁺ T cells prevents bronchial eosinophilia and hyperresponsiveness in a murine asthma model (3). CD8⁺ T cells seem to play a protective role in asthma because depletion upregulates (4) and administration of antigen-primed CD8⁺ cells suppresses both allergen-induced hyperresponsiveness and eosinophilic inflammation (5). On the other hand, CD8⁺ T cells are found to be essential for the influx of eosinophils into the lung and the development of airway hyperresponsiveness (AHR) in response to respiratory syncytial virus infection (6). The role of $\gamma\delta$ T cells in asthma is not well defined and there is an ongoing debate as to whether they

are essential or protective. Whereas some authors favor a key role of $\gamma\delta$ T cells for the development of allergic airway inflammation (7–9), others have demonstrated a down-regulatory effect on AHR and immunoglobulin (Ig) E production (10–12).

Regarding the cytokine expression of pulmonary T cells, it has been well established over recent years that asthmatic airway inflammation is characterized by an increased expression of the T helper (Th)2-type cytokines interleukin (IL)-4, IL-5, and IL-13 (13–16). These cytokines are of major importance because IL-4 and IL-13 induce the production of IgE by B cells and IL-5 regulates the growth, differentiation, and activation of eosinophils (17). The role of the Th1 cytokine interferon (IFN)- γ in asthma is still a matter of debate: in an earlier study we described an increased frequency of IFN- γ ⁺ T cells in bronchoalveolar lavage fluid (BALF) from asthmatic compared with control subjects (18), and Hessel and colleagues have clearly demonstrated that the development of AHR is IFN- γ -dependent (19). However, other investigators have shown an inhibitory effect of IFN- γ on pulmonary allergic responses (20).

Although T cells are well accepted as regulatory cells in allergic airway inflammation through the release of cytokines, only limited information is available on the phenotype of the cytokine-expressing T cells: Ying and associates (21) have shown that IL-4 and IL-5 messenger RNA signals were colocalized to CD4⁺ cells and, to a lesser extent, to CD8⁺ cells. BALF-derived CD4⁺ and CD8⁺ cell lines from asthmatic subjects released elevated levels of IL-5 when compared with controls (22). Regarding $\gamma\delta$ ⁺ T cells, Spinozzi and coworkers demonstrated increased amounts of intracellular IL-4 in isolated $\gamma\delta$ T cells from BALF of asthmatic subjects (23).

The aim of the present study was therefore to investigate systematically the cytokine profiles of pulmonary T-cell subsets. We determined the proportion of CD4⁺, CD8⁺, and $\gamma\delta$ ⁺ T cells from BALF that expressed the Th1-type cytokines IFN- γ and IL-2 and the Th2-type cytokines IL-4, IL-5, and IL-13 using a flow cytometric intracellular cytokine assay. BALF was obtained from mild atopic asthmatic and nonatopic control subjects at baseline and 24 h after segmental allergen and saline challenge.

(Received in original form March 31, 2000 and in revised form December 27, 2000)

Address correspondence to: Norbert Krug, M.D., Fraunhofer-Institute of Toxicology and Aerosol Research, Dept. of Immunology, Allergology and Clinical Inhalation, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany. E-mail: krug@ita.fhg.de

Abbreviations: Airway hyperresponsiveness, AHR; bronchoalveolar lavage fluid, BALF; interferon, IFN; immunoglobulin, Ig; interleukin, IL; phosphate-buffered saline, PBS; phycoerythrin, PE; T-cell receptor, TCR; T helper, Th.

Am. J. Respir. Cell Mol. Biol. Vol. 25, pp. 125–131, 2001
Internet address: www.atsjournals.org

Materials and Methods

Study Subjects

A total of 11 patients with mild asthma and nine normal control subjects participated in the study (for patient characteristics, see Table 1). All patients had mild allergic asthma as defined in the Consensus Report (24). They had a history of intermittent 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 > 4-mm-diameter skin-

wheal response to one or more of eight common allergens (*Der-matophagoides pteronyssinus*, *D. farinae*, mixed grass pollen, mixed tree pollen, dog hair, feather, cat fur, and *Alternaria*; allergen extraction from Abelló, Bornheim, Germany). The allergen extract used for segmental allergen challenge (mixed grass pollen or *D. pteronyssinus*; Abelló) was that which produced the largest wheal response on skin-prick testing, and the chosen concentration was one-tenth the dilution in saline that elicited a 3-mm-diameter skin-wheal response. The instilled amount of antigen was 0.01 to 0.1 µg for mixed grass pollen (a mixture of the major allergens Dac g5, Fes p5, Lol p5, Phl p5, and Poa p5) and 0.6 µg for *D. pteronyssinus* (a mixture of the major allergens Der p1 and Der p2). Bronchial hyperresponsiveness and PC₂₀FEV₁ were determined as described (25). Patients were using salbutamol only when required for relief of symptoms. No patients were treated with corticosteroids, sodium cromoglycate, theophylline, or leukotriene-modifying drugs. The normal control subjects had no history of allergic or other diseases, showed negative skin-prick tests, had normal IgE (≤ 100 IU/ml) and normal lung function tests, and had no bronchial hyperresponsiveness (PC₂₀ > 8 mg/ml). All study subjects were nonsmokers and no subject had an acute bronchitis 4 wk before the investigations. All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the studies, which were approved by the Ethical Committee of Hannover Medical School.

Segmental Allergen Challenge

Segmental allergen challenge was performed as previously described (25, 26). A commercially available endotoxin detection assay (E-toxate, *Limulus* amoebocyte lysate; Sigma, St. Louis, MO)

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 subcutaneously), and midazolam (mean 4.8 mg intravenously, range 0 to 8 mg) before the bronchoscopy. Lidocaine was used to achieve local anesthesia of upper and lower airways. The bronchoscope (P30; Olympus Optical, Tokyo, Japan) was wedged into the inferior lingular bronchus and bronchoalveolar lavage was performed with 5 × 20 ml sterile saline. The instrument was passed into the superior lingular bronchus and 10 ml saline solution was instilled as a control challenge. Finally, the bronchoscope was passed to the medial segment of the middle lobe and 10 ml allergen solution was instilled. After 24 h, subjects (all of the asthmatic and five of the control subjects) were rebronchoscoped with the same premedication, and the superior lingular bronchi and the medial middle bronchi were lavaged with 100 ml saline.

BALF samples were processed as described (25). Briefly, cells were filtered through a 100-µm filter, centrifuged at 250 × g for 10 min, and washed in phosphate-buffered saline (PBS). The total count of nucleated cells was performed using a Neubauer hemocytometer. Differential cell counts were performed from cytospin slides, with 300 cells per slide being counted. An aliquot of the cells was separated for flow cytometric analysis.

Flow Cytometric Detection of Intracellular Cytokines in BALF T Cells

Intracellular cytokine detection of BALF-derived T cells was performed as previously described (18). Briefly, BALF cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) (1×10^6 cells/ml). Cells were

TABLE 1
Clinical characteristics of the study subjects

Subjects	Age	Sex	FEV ₁ (%)	PC ₂₀ FEV ₁ Histamin (mg/ml)	IgE (kU/liter)	Allergen Sensitivity*	Allergen Provocation†
Control	33	F	103	10.2	62	—	—
	32	M	95	11.9	5	—	—
	23	M	103	10.5	52	—	—
	27	M	101	20.0	7	—	—
	23	M	104	25.4	7	—	0.1 µg gp
	25	M	108	11.9	3	—	0.1 µg gp
	24	M	103	11.3	9	—	0.1 µg gp
	28	M	96	16.1	8	—	0.6 µg Dp
	33	F	104	20.1	8	—	0.6 µg Dp
median	27	2F/7M	103	11.9	8		
(range)	(23–33)		(95–108)	(10.2–25.4)	(3–62)		
Asthmatic	22	M	91	12.4	310	gp, hp, Al, c	0.1 µg gp
	29	M	101	5.2	390	gp, Dp, Df, d	0.1 µg gp
	22	F	100	5.6	415	gp, tp, Al, Cl, c	0.01 µg gp
	27	M	109	2.9	323	gp, hp, tp, Al	0.1 µg gp
	28	M	99	8.4	110	gp, tp, Al	0.1 µg gp
	26	M	96	3.6	73	gp, Al, c	0.1 µg gp
	24	M	90	8.5	168	gp, tp, Dp, Al, c	0.1 µg gp
	26	M	119	4.1	194	gp, hp, Dp, Df, c	0.1 µg gp
	23	F	79	0.5	96	Gp, Dp, Df, K	0.1 µg gp
	22	M	115	13.0	40	tp, Dp, Df, c	0.6 µg Dp
	28	F	109	1.0	78	gp, tp, Dp, Df, Al, Cl, c	0.1 µg gp
median	26	3F/8M	100	5.2	168		
(range)	(22–29)		(79–119)	(0.5–13.0)*	(40–415)*		

*Allergen sensitivity was determined by skin-prick test (gp, mixed grass pollen; hp, mixed herb pollen; tp, mixed tree pollen; Dp, *D. pteronyssinus*; Df, *D. farinae*; Al, *Alternaria*; Cl, *Cladosporia*; c, cat fur; d, dog hair).

†Allergen provocation was done with either grass pollen mix (gp; Dac g5, Fes p5, Lol p5, Phl p5, Poa p5) or *D. pteronyssinus* mix (Dp; Der p1, Der p2).

‡P < 0.01 compared with control subjects.

TABLE 2
Basic BALF data

Subjects	BALF	n	Recovery (ml)	Total Cells ($\times 10^6$)	Eosinophils		Neutrophils		Lymphocytes		Macrophages	
					($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)	($\times 10^6$)	(%)
Control	B	9	77	7.3	0.0	0.0	0.1	1.0	0.3	5.0	7.0	93.0
			(58–88)	(3.0–25.5)	(0.0–0.1)	(0.0–0.5)	(0.0–0.1)	(0.0–2.0)	(0.2–0.7)	(1.0–10.0)	(2.7–25.0)	(89.0–98.0)
	S	5	76	12.0	0	0	2.8	23.0	0.4	3.0	8.6	72.0
			(72–82)	(3.0–75.0)			(0.1–42.8)*	(2.0–57.0)*	(0.2–1.5)	(2.0–6.0)*	(2.8–30.8)	(41.0–92.0)*
	A	5	78	17.4	0	0	4.7	30.0	0.9	4.0	11.8	68.0
			(60–90)	(6.0–126.0)			(0.2–65.5)*	(4.0–52.0)*	(0.3–5.0)	(2.0–7.0)	(5.3–55.4)	(44.0–89.0)*
Asthma	B	11	74	10.0	0.0	0.0	0.0	0.5	0.5	6.0	8.8	92.0
			(60–90)	(4.1–15.0)	(0.0–0.5)	(0.0–3.0)	(0.0–0.3)	(0.0–3.0)	(0.1–4.5)	(1.0–39.4)	(3.8–14.4)	(59.0–96.0)
	S	11	71	19.2	0.0	0.3	12.8	6.3	0.8	4.3	12.8	71.0
			(46–86)	(3.9–110.0)*	(0.0–3.3)	(0.0–3.0)	(2.7–89.8) [†]	(0.2–23.3) [†]	(0.0–10.2)	(2.0–17.0)	(2.7–89.8)	(21.0–95.4) [†]
	A	11	71	28.0	7.3	28.0	2.5	8.0	0.8	3.0	10.0	38.0
			(58–140)	(4.6–364.0)*	(0.2–218.3) ^{††}	(1.0–85.6) ^{††}	(0.2–262.1) [†]	(2.0–72.0) [†]	(0.1–18.6)	(2.0–7.3)*	(2.1–80.1)	(7.0–86.0) [†]

Fluid recovery, cell counts, and percentage of cells in BALF from healthy control and asthmatic subjects.

BALF was obtained at baseline (B) and 24 h after saline challenge (S) or allergen challenge (A). Values are given as median and range.

* and [†] indicate $P < 0.05$ and $P < 0.01$, respectively, compared with baseline values.

^{††}indicates $P < 0.01$ compared with controls.

cultured in 24-well flat-bottom plates (Nunc, Wiesbaden, Germany) and stimulated with phorbol 12-myristate 13-acetate (PMA; 10 ng/ml) and ionomycin (1 μ M) in the presence of monensin (2.5 μ M). After incubation for 4 h at 37°C in a humidified atmosphere of 5% CO₂ in air, cells were washed in PBS and half of the cells were fixed in 1 ml 4% ice-cold paraformaldehyde (Riedel de Haen, Seelze, Germany) for 10 min. After a further wash in PBS, these cells were resuspended in 100- μ l portions with saponin buffer (PBS containing 0.1% saponin [Riedel de Haen] and 0.01 M *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid buffer [Serva, Heidelberg, Germany]). Anti-CD4-fluorescein isothiocyanate (FITC) (1 μ g/ml; Coulter, Krefeld, Germany), anti-CD8-Tricolor (1 μ g/ml; Medac, Hamburg, Germany), and phycoerythrin (PE)-labeled antibodies against IFN- γ , IL-2, IL-4, IL-5, and IL-13 (10 μ l, dilution 1:30; Pharmingen, San Diego, CA) were added to the cell suspension and incubated for 30 min at 4°C in the dark. The second half of the cells was first stained with anti-T-cell receptor (TCR)- $\gamma\delta$ -FITC (1 μ g/ml; Becton Dickinson, Heidelberg, Germany) and anti-CD3-Tricolor (1 μ g/ml; Medac) for 30 min before fixation after staining with PE-labeled antibodies against IFN- γ , IL-2, IL-4, IL-5, and IL-13 in saponin buffer as described earlier. With this technical modification the expression of the TCR- $\gamma\delta$ was well detectable, whereas its expression was markedly downregulated after the fixation. After a final wash in saponin buffer, cells were resuspended in PBS and kept in the dark at 4°C until flow cytometric evaluation. As controls, nonspecific FITC-, PE-, and Tricolor-labeled antibodies were used (Becton Dickinson and Medac). Flow cytometric analysis was performed with scatter gates on the lymphocyte fraction using a flow cytometer (Calibur; Becton Dickinson). The percentages of CD3⁺, CD4⁺, CD8⁺, and TCR- $\gamma\delta$ ⁺ lymphocytes were calculated in the FI-1/FI-3 dot blot. The percentages of cytokine-positive CD4⁺ and TCR- $\gamma\delta$ ⁺ lymphocytes were calculated in the FI-1/FI-2 dot blot, and the percentage of CD8⁺ lymphocytes in the FI-2/FI-3 dot blot. 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 (PE). Cells that were preincubated with recombinant cytokines before staining with anticytokine antibodies and cells that were not stimulated *in vitro* did not show positive cytokine staining. If not indicated differently, reagents were purchased from Sigma.

Statistical Analysis

The Mann–Whitney U test was used for intergroup comparison between asthmatic and control subjects. For comparison of paired data within the major study groups (baseline, saline, and allergen) the Wilcoxon test was used for the individual comparisons. The Bonferroni correction was used throughout. Probability values of $P < 0.05$ were accepted as significant.

Results

BALF Recovery, Differential Cell Count, and T-Cell Subsets

BALF recovery did not differ between baseline, saline-challenged, and allergen-challenged BALF (Table 2). There was an increased total cell count after saline and allergen chal-

TABLE 3
Lymphocyte subpopulations in BALF

Subjects	BALF	n	CD3 ⁺ (%)	CD4 ⁺ (%)	CD8 ⁺ (%)	TCR- $\gamma\delta$ ⁺ (%)
Control	B	9	92.2	53.4	38.5	2.5
			(88.5–95.8)	(37.7–79.6)	(17.1–48.5)	(1.2–3.9)
	S	5	92.8	59.7	31.1	2.5
			(91.2–97.1)	(38.4–75.8)	(16.8–47.6)	(1.5–3.5)
	A	5	90.7	54.6	28.9	2.3
			(89.5–95.1)	(49.3–80.5)	(15.2–36.7)*	(1.3–4.3)
Asthma	B	11	92.5	56.6	35.2	2.5
			(91.1–96.8)	(25.6–81.2)	(15.0–69.0)	(1.4–9.0)
	S	11	91.1	62.5	30.0	2.5
			(81.9–97.4)	(36.0–76.3)	(14.6–54.7)	(1.6–10.0)
	A	11	83.4	67.5	23.0	1.9
			(75.2–93.4) ^{††}	(35.7–81.0)	(13.4–44.2)	(0.7–12.7)

Percentages of lymphocyte subpopulations in BALF from healthy control and asthmatic subjects.

BALF was obtained at baseline (B) and 24 h after saline challenge (S) or allergen challenge (A). Values are given as median and range.

* and [†] indicate $P < 0.05$ and $P < 0.01$, respectively, compared with baseline values.

^{††}indicates $P < 0.01$ compared with controls.

lunge in asthmatic and control subjects that did achieve statistical significance only in the asthmatic subjects ($P < 0.05$). The absolute numbers and the percentages of neutrophils were increased after saline and allergen challenge in both asthmatic ($P < 0.01$) and control ($P < 0.05$) subjects, whereas the numbers and percentages of eosinophils were elevated only after allergen challenge in asthmatic subjects ($P < 0.01$). There was a small decrease in the percentage of lymphocytes after saline challenge in the con-

trol subjects ($P < 0.05$) and after allergen challenge in the asthmatic subjects ($P < 0.05$). However, the absolute numbers did not change after the challenges. There was a decrease in the percentage of CD3⁺ cells after allergen challenge in asthmatic ($P < 0.01$) and of CD8⁺ cells after allergen challenge in control ($P < 0.05$) subjects. The percentages of CD4⁺ and $\gamma\delta$ ⁺ T cells were not different between asthmatic and control subjects and did not change after the challenges (Table 3).

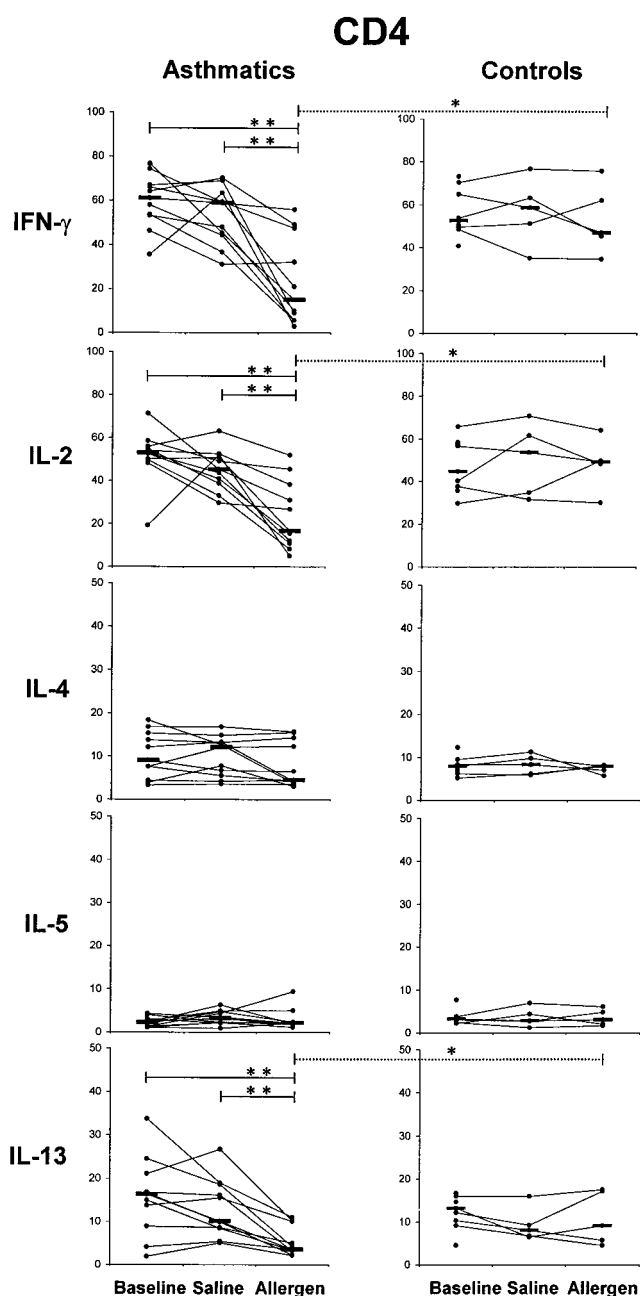


Figure 1. Percentages of CD4⁺ lymphocytes with positive intracellular staining for IFN- γ , IL-2, IL-4, IL-5, and IL-13 in BALF from asthmatic ($n = 11$) and control ($n = 9$) subjects. BALF was obtained at baseline and 24 h after segmental saline and allergen challenge. Bars indicate median values. * $P < 0.05$; ** $P < 0.01$.

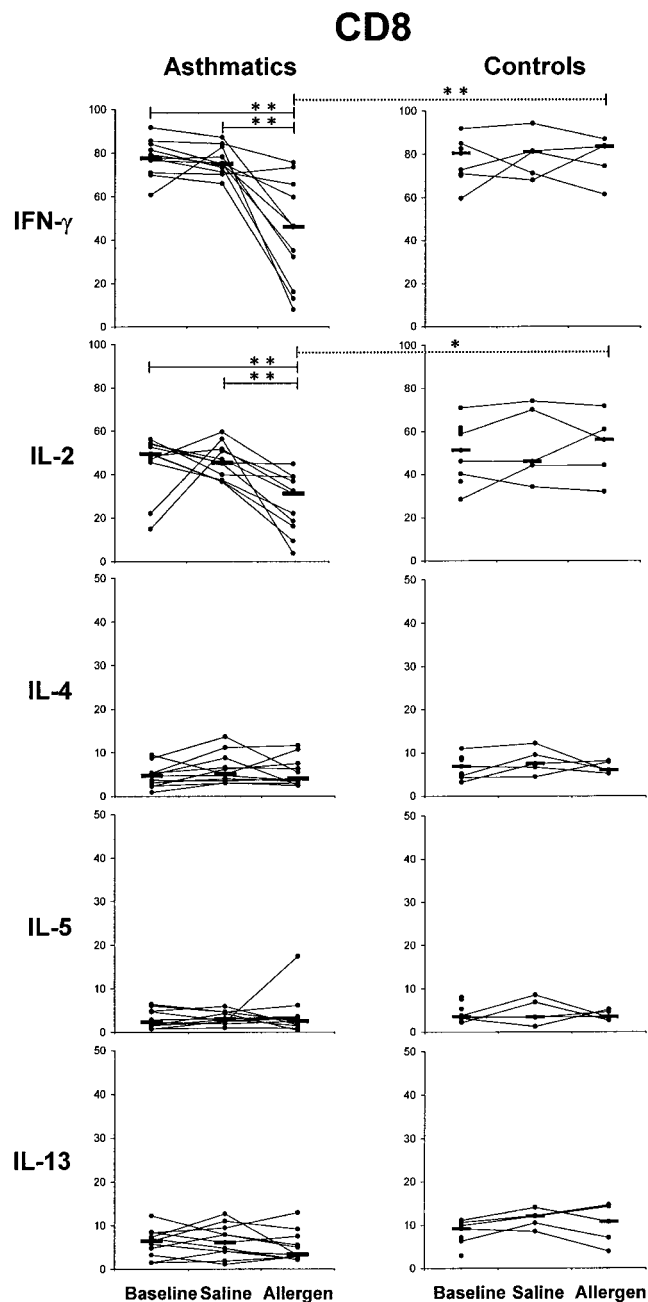


Figure 2. Percentages of CD8⁺ lymphocytes with positive intracellular staining for IFN- γ , IL-2, IL-4, IL-5, and IL-13 in BALF from asthmatic ($n = 11$) and control ($n = 9$) subjects. BALF was obtained at baseline and 24 h after segmental saline and allergen challenge. Bars indicate median values. * $P < 0.05$; ** $P < 0.01$.

Intracellular Expression of Cytokines in BALF T Cells

Figures 1–3 show the percentages of CD4⁺, CD8⁺, and $\gamma\delta$ ⁺ T cells staining positively for the cytokines IFN- γ , IL-2, IL-4, IL-5, and IL-13 after 4 h of stimulation with PMA and ionomycin in the presence of monensin. Although at baseline there was a high percentage of IFN- γ - and IL-2-staining cells in all T-cell subsets in asthmatic and control subjects, IL-4, IL-5, and IL-13 were produced only by a small percentage of T cells. The major difference between

asthmatic and control subjects at baseline was an increased percentage of IL-5- and IL-13-producing $\gamma\delta$ ⁺ T cells in asthmatic subjects ($P < 0.05$). After allergen, but not after saline, challenge there was a significant decrease in the percentages of IFN- γ - and IL-2-staining cells for all subsets in asthmatic subjects ($P < 0.01$), whereas the percentages of IL-4- and IL-5-producing T cells did not change. For IL-13-producing T cells there was a decrease after allergen challenge only in the CD4⁺ subset ($P < 0.01$). In control subjects, no changes in the percentages of cytokine-producing T cell were detectable for all subsets after allergen or saline challenge.

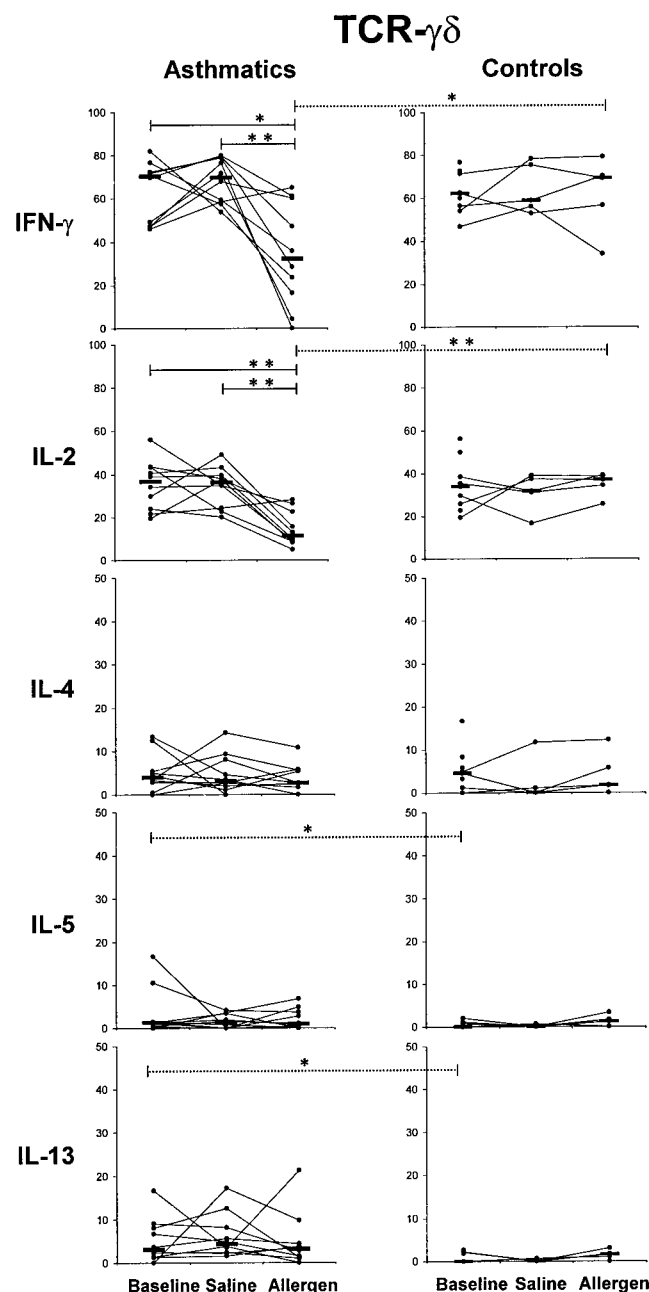


Figure 3. Percentages of $\gamma\delta$ T cells with positive intracellular staining for IFN- γ , IL-2, IL-4, IL-5, and IL-13 in BALF from asthmatic ($n = 11$) and control ($n = 9$) subjects. BALF was obtained at baseline and 24 h after segmental saline and allergen challenge. Bars indicate median values. * $P < 0.05$; ** $P < 0.01$.

Discussion

The aim of the present study was to investigate the capacity of T-cell subsets in the BALF of patients with mild asthma to produce the Th1-type cytokines IFN- γ and IL-2 and the Th2-type cytokines IL-4, IL-5, and IL-13.

First, we determined in atopic asthmatic, compared with nonatopic control, subjects the percentages of CD4⁺, CD8⁺, or $\gamma\delta$ T cells before and after segmental allergen or saline challenge. In agreement with other investigators (27–29), we found basically no changes in the proportions of CD4⁺ and CD8⁺ T cells between the groups and after the provocation. Regarding $\gamma\delta$ T cells, the results are conflicting: Spinozzi and colleagues showed an increased proportion of $\gamma\delta$ T cells in BALF from asthmatic compared with control subjects (7), whereas other investigators have shown, in agreement with our results, no differences in the numbers of $\gamma\delta$ T cells in BALF (30) or bronchial biopsies (31). A possible explanation for these discrepancies might be differences in the degrees of asthma severity. Our study included only subjects with very mild asthma. Further, the time after allergen challenge might be critical for determination when and if an expansion of $\gamma\delta$ T cells is seen. Whereas we looked at a rather early time point after allergen challenge, the other studies investigated stable asthmatic subjects without a definite allergen provocation.

Although we found no changes in the T-cell subset distribution, functional differences regarding their cytokine profiles were prominent: the major difference between asthmatic and control subjects before allergen challenge was an increased percentage of $\gamma\delta$ T cells with positive staining for the Th2 cytokines IL-5 and IL-13. These data suggest that in asthma there is a small but significant subset of pulmonary $\gamma\delta$ T cells that produce Th2-type cytokines and might control the Th2-driven allergic inflammation. It has been shown in the past that the number of Th2-type-producing cells is not necessarily the most relevant factor because, for example, very low numbers of CD4⁺ NK1.1⁺ cells can produce large amounts of Th2-type cytokines (32) and depletion of these cells reduces the allergic inflammation (33). Our data are of particular interest in relation to a recent study in a mouse asthma model, which has shown that the development of Th2-mediated allergic airway inflammation is dependent on the presence of $\gamma\delta$ T cells (9). In this study the decrease in specific IgE, T-cell and eosinophil infiltration, and pulmonary IL-5 levels in $\gamma\delta$ T cell-deficient mice was restored by administration of IL-4 during the primary immunization, indicating that $\gamma\delta$

T cell–derived Th2 cytokines might be a key step in the development and maintenance of the allergic reaction. Schramm and associates confirmed the proinflammatory role of $\gamma\delta$ T cells in a similar animal model (34) and demonstrated a reduced AHR in allergen-challenged, $\gamma\delta$ T cell–deficient mice. Although we did not find increased percentages of IL-4–producing $\gamma\delta$ T cells, Spinazzi and co-workers demonstrated increased amounts of intracellular IL-4 in isolated $\gamma\delta$ T cells from BALF of asthmatic subjects (23). Further, increased percentages of IL-4– and IL-5–producing $\gamma\delta$ T cells were described in the nasal mucosa of allergic patients (35). The most prominent finding in our study was the increased percentage of IL-13–producing $\gamma\delta$ T cells in asthmatic subjects. The Th2-type cytokine IL-13 has recently been shown to be a key cytokine for the development of the asthmatic phenotype, independently of IL-4 (36–38). Thus, together with the fact that $\gamma\delta$ T cells clearly can differentiate into Th2 cells (39, 40), these data suggest that pulmonary $\gamma\delta$ T cells might play a proinflammatory role in allergic asthma through the release of Th2-type cytokines.

In contrast to these findings, Jaffar and associates demonstrated a lack of IL-5 production by stimulation of bronchial biopsies with anti-TCR- $\gamma\delta$, whereas stimulation with allergen, anti-CD3, and anti-TCR- $\alpha\beta$ showed increased IL-5 production in biopsies from asthmatic subjects (41). These findings argue against a release of Th2-type cytokines from $\gamma\delta$ T cells. Further, a recent report has demonstrated a novel suppressive effect of $\gamma\delta$ T cells on AHR in a murine asthma model: Lahn and colleagues (12) have shown in $\gamma\delta$ T cell–deficient mice that despite a reduction in eosinophilic airway inflammation the AHR in these mice was increased after inhalation allergen challenge. Although the precise target for the regulatory $\gamma\delta$ T cells remains to be defined, the authors conclude that $\gamma\delta$ T cells might play a role in the epithelial repair mechanism, which can suppress the development of bronchial hyperresponsiveness (42). These at-first-glance contradictory results might be explained by multiple roles $\gamma\delta$ T cells could play in bronchial inflammation. It might well be that $\gamma\delta$ T cells that occur early in the inflammatory response have a proinflammatory role, whereas a different subset occurring several days later is involved in epithelial repair and plays an anti-inflammatory role.

After segmental allergen challenge, CD4⁺, CD8⁺, and $\gamma\delta$ T-cell subsets showed a uniform pattern of reduced ability to produce the Th1-type cytokines IFN- γ and IL-2 in asthmatic but not in control subjects, whereas the percentages of IL-4– and IL-5–positive cells did not change. These data suggest that in asthmatic subjects after allergen exposure the balance between Th1- and Th2-type cytokines is skewed toward Th2 production. Interestingly, all three subsets contributed uniformly to these changes, with the exception of IL-13 production: only CD4⁺ cells showed a reduced percentage of IL-13–positive cells after allergen challenge, which is difficult to explain in this context. The decreased Th1 cytokine staining might be partly due to an activation-induced apoptosis; we have previously demonstrated increased Fas ligand expression at least on BALF-derived CD4⁺ and CD8⁺ cells after allergen challenge (25). Regarding the expression of IFN- γ , the results from a

previous study are partially in contrast to the present data: we have shown previously in BALF T cells from asthmatic subjects, compared with control subjects, an increased capacity to produce IFN- γ (18), whereas in the present study, under baseline conditions, all T-cell subsets from both groups did not differ in their ability to produce IFN- γ . A possible explanation for the discrepancy might be the more severe asthmatic subjects in the previous investigation as compared with the patients with very mild asthma in the present study. However, the decrease in IFN- γ –producing cells after allergen challenge was not expected. This finding is, on the other hand, in agreement with results from other investigators who have demonstrated decreased IFN- γ concentration in BALF 18 h after segmental allergen challenge (27). Because IFN- γ is primarily produced by lymphocytes, these data are consistent. However, in the same study and in a follow-up study (16) IL-2, IL-4, IL-5, and IL-13 concentrations in BALF were increased after segmental allergen challenge, which seems to be contradictory to our results. Because IL-4, IL-5, and IL-13 can be produced by a variety of non-T cells, including mast cells, eosinophils, and basophils, elevated cytokine concentrations can be well explained despite unchanged or reduced numbers of cytokine-producing T cells. Further, one important limitation of the flow cytometric method used is the need for *in vitro* stimulation of the cells. Therefore, only the potential for cytokine production upon polyclonal *in vitro* activation can be determined. A modification of this method using immunocytochemistry has been described, which found a generally similar pattern in the percentages of IFN- γ –, IL-4–, and IL-5–producing T cells in asthmatic subjects (43). Because this method is more sensitive in detecting cytokine-producing cells it might have the potential to determine cytokine production after allergen challenge without stimulation.

In conclusion, pulmonary $\gamma\delta$ T cells from stable asthmatic subjects showed an increased capacity to produce Th2-type cytokines upon stimulation, whereas CD4⁺, CD8⁺, and $\gamma\delta$ T cells revealed a reduced potential to secrete Th1-type cytokines after segmental allergen challenge. These data support the hypothesis that in allergic asthma a dysbalance between the expression of T cell–derived Th1-type and Th2-type cytokines exists, which is skewed toward the Th2-type. Because the T-cell cytokine production in the present study was determined after polyclonal stimulation, future research should clarify the allergen-specific cytokine production of T-cell subsets, which would ideally be possible with a modification of the cytokine assay used here, already applied in animal models (44).

Acknowledgments: This study was supported by a research grant from the Deutsche Forschungsgemeinschaft (Kr 1405/2-1).

References

1. Djukanovic, R., W. R. Roche, J. W. Wilson, C. R. W. Beasley, P. Twentyman, P. H. Howarth, and S. T. Holgate. 1990. Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.* 142:434–457.
2. Wilson, J. W., R. Djukanovic, P. H. Howarth, and S. T. Holgate. 1992. Lymphocyte activation in bronchoalveolar lavage and peripheral blood in atopic asthma. *Am. Rev. Respir. Dis.* 145:958–960.
3. Gavett, S. H., X. Chen, F. Finkelman, and M. Wills-Karp. 1994. Depletion of murine CD4⁺ T lymphocytes prevents antigen-induced airway hyperactivity and pulmonary eosinophilia. *Am. J. Respir. Cell Mol. Biol.* 10:587–593.

4. Huang, T. J., P. A. MacAry, D. M. Kemeny, and K. F. Chung. 1999. Effect of CD8+ T-cell depletion on bronchial hyper-responsiveness and inflammation in sensitized and allergen-exposed Brown-Norway rats. *Immunology* 96:416–423.
5. Suzuki, M., R. Taha, D. Ihaku, Q. Hamid, and J. G. Martin. 1999. CD8+ T cells modulate late allergic airway responses in Brown Norway rats. *J. Immunol.* 163:5574–5581.
6. Schwarze, J., G. Cieslewicz, A. Joetham, T. Ikemura, E. Hamelmann, and E. W. Gelfand. 1999. CD8 T cells are essential in the development of respiratory syncytial virus-induced lung eosinophilia and airway hyperresponsiveness. *J. Immunol.* 162:4207–4211.
7. Spinozzi, F., E. Agea, O. Bistoni, N. Forenza, A. Monaco, G. Bassotti, I. Nicoletti, C. Riccardi, F. Grignani, and A. Bertotto. 1996. Increased allergen-specific, steroid-sensitive gamma delta T cells in bronchoalveolar lavage fluid from patients with asthma. *Ann. Intern. Med.* 124:223–227.
8. Spinozzi, F., E. Agea, O. Bistoni, N. Forenza, and A. Bertotto. 1998. Gamma delta T cells, allergen recognition and airway inflammation. *Immunol. Today* 19:22–26.
9. Zuany-Amorim, C., C. Ruffie, S. Haile, B. B. Vargaftig, P. Pereira, and M. Pretolani. 1998. Requirement for gammadelta T cells in allergic airway inflammation. *Science* 280:1265–1267.
10. McMenamin, C., C. Pimm, M. McKersy, and P. G. Holt. 1994. Regulation of IgE responses to inhaled antigen in mice by antigen-specific gamma delta T cells. *Science* 265:1869–1871.
11. McMenamin, C., M. McKersy, P. Kuhnlein, T. Hunig, and P. G. Holt. 1995. Gamma delta T cells down-regulate primary IgE responses in rats to inhaled soluble protein antigens. *J. Immunol.* 154:4390–4394.
12. Lahn, M., A. Kanehio, K. Takeda, A. Joetham, J. Schwarze, G. Kohler, R. O'Brien, E. W. Gelfand, and W. Born. 1999. Negative regulation of airway responsiveness that is dependent on gammadelta T cells and independent of alphabeta T cells [see comments]. *Nat. Med.* 5:1150–1156.
13. Robinson, D. S., Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A. M. Bentley, C. Corrigan, S. Durham, and A. B. Kay. 1992. Predominant Th2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N. Engl. J. Med.* 326:298–304.
14. Humbert, M., S. R. Durham, S. Ying, P. Kimmitt, J. Barkans, B. Assoufi, R. Pfister, G. Menz, D. S. Robinson, A. B. Kay, and C. J. Corrigan. 1996. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity. *Am. J. Respir. Crit. Care Med.* 154:1497–1504.
15. Humbert, M., S. R. Durham, P. Kimmitt, N. Powell, B. Assoufi, R. Pfister, G. Menz, A. B. Kay, and C. J. Corrigan. 1997. Elevated expression of messenger ribonucleic acid encoding IL-13 in the bronchial mucosa of atopic and nonatopic subjects with asthma. *J. Allergy Clin. Immunol.* 99:657–665.
16. Kroegel, C., P. Julius, H. Matthys, J. C. Virchow, Jr., and W. Luttmann. 1996. Endobronchial secretion of interleukin-13 following local allergen challenge in atopic asthma: relationship to interleukin-4 and eosinophil counts. *Eur. Respir. J.* 9:899–904.
17. Chung, K. F., and P. J. Barnes. 1999. Cytokines in asthma. *Thorax* 54:825–857.
18. Krug, N., J. Madden, A. E. Redington, P. Lackie, R. Djukanovic, U. Schauer, S. T. Holgate, A. J. Frew, and P. H. Howarth. 1996. T cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 14:319–326.
19. Hessel, E. M., A. J. Van Oosterhout, I. Van Ark, B. Van Esch, G. Hofman, H. Van Loveren, H. F. Savelkoul, and F. P. Nijkamp. 1997. Development of airway hyperresponsiveness is dependent on interferon-gamma and independent of eosinophil infiltration. *Am. J. Respir. Cell Mol. Biol.* 16:325–334.
20. Li, X. M., R. K. Chopra, T. Y. Chou, B. H. Schofield, M. Wills-Karp, and S. K. Huang. 1996. Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice. *J. Immunol.* 157:3216–3219.
21. Ying, S., M. Humbert, J. Barkans, C. J. Corrigan, R. Pfister, G. Menz, M. Larché, D. S. Robinson, S. R. Durham, and A. B. Kay. 1997. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J. Immunol.* 158:3539–3544.
22. Till, S., B. Li, S. Durham, M. Humbert, B. Assoufi, D. Huston, R. Dickason, P. Jeannin, A. B. Kay, and C. Corrigan. 1995. Secretion of the eosinophil-active cytokines interleukin-5, granulocyte/macrophage colony-stimulating factor and interleukin-3 by bronchoalveolar lavage CD4+ and CD8+ T cell lines in atopic asthmatics, and atopic and non-atopic controls. *Eur. J. Immunol.* 25:2727–2731.
23. Spinozzi, F., E. Agea, O. Bistoni, N. Forenza, A. Monaco, B. Falini, G. Bassotti, F. De Benedictis, F. Grignani, and A. Bertotto. 1995. Local expansion of allergen-specific CD30+Th2-type gamma delta T cells in bronchial asthma. *Mol. Med.* 1:821–826.
24. National Heart, Lung, and Blood Institute. 1992. International Consensus Report on Diagnosis and Treatment of Asthma. NHLBI publication no. 92-3091. *Eur. Respir. J.* 5:601–641.
25. Krug, N., T. Tschernig, K. Balke, V. J. Erpenbeck, L. Meyer, U. Bode, J. M. Hohlfeld, R. Pabst, and H. Fabel. 1999. Enhanced expression of fas ligand (CD95L) on T cells after segmental allergen provocation in asthma. *J. Allergy Clin. Immunol.* 103:649–655.
26. Krug, N., L. M. Teran, A. E. Redington, C. Gratziau, S. Montefort, R. Polosa, H. Brewster, P. H. Howarth, S. T. Holgate, A. J. Frew, and M. P. Carroll. 1996. Safety aspects of local endobronchial allergen challenge in asthmatic patients. *Am. J. Respir. Crit. Care Med.* 153:1391–1397.
27. Virchow, J. C., Jr., C. Walker, D. Hafner, C. Kortsik, P. Werner, H. Matthys, and C. Kroegel. 1995. T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma. *Am. J. Respir. Crit. Care Med.* 151:960–968.
28. Frew, A. J., J. St-Pierre, L. M. Teran, A. Trefiliev, J. Madden, D. Peroni, K. M. Bodey, A. F. Walls, P. H. Howarth, M. P. Carroll, and S. T. Holgate. 1996. Cellular and mediator responses twenty-four hours after local endobronchial allergen challenge of asthmatic airways. *J. Allergy Clin. Immunol.* 98:133–143.
29. Kelly, E. A., R. R. Rodriguez, W. W. Busse, and N. N. Jarjour. 1997. The effect of segmental bronchoprovocation with allergen on airway lymphocyte function. *Am. J. Respir. Crit. Care Med.* 156:1421–1428.
30. Walker, C., M. K. Kaegi, P. Braun, and K. Blaser. 1991. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J. Allergy Clin. Immunol.* 88:935–942.
31. Fajac, I., G. L. Roisman, J. Lacronique, B. S. Polla, and D. J. Dusser. 1997. Bronchial gamma delta T-lymphocytes and expression of heat shock proteins in mild asthma. *Eur. Respir. J.* 10:633–638.
32. Yoshimoto, T., and W. E. Paul. 1994. CD4+, NK1.1+ T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J. Exp. Med.* 179:1285–1295.
33. Walker, C., J. Checkel, S. Cammisuli, P. J. Leibson, and G. J. Gleich. 1998. IL-5 production by NK cells contributes to eosinophil infiltration in a mouse model of allergic inflammation. *J. Immunol.* 161:1962–1969.
34. Schramm, C. M., L. Puddington, C. A. Yamouyannis, E. G. Lingenheld, H. E. Whiteley, W. W. Wolyniec, T. C. Noonan, and R. S. Thrall. 2000. Proinflammatory roles of T-cell receptor (TCR) gammadelta and TCR alphabeta lymphocytes in a murine model of asthma. *Am. J. Respir. Cell Mol. Biol.* 22:218–225.
35. Pawankar, R. U., M. Okuda, K. Suzuki, K. Okumura, and C. Ra. 1996. Phenotypic and molecular characteristics of nasal mucosal gamma delta T cells in allergic and infectious rhinitis. *Am. J. Respir. Crit. Care Med.* 153:1655–1665.
36. Vogel, G. 1998. Interleukin-13's key role in asthma shown. *Science* 282:2168.
37. Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282:2261–2263.
38. Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282:2258–2261.
39. Ferrick, D. A., M. D. Schrenzel, T. Mulvanian, B. Hsieh, W. G. Ferlin, and H. Lepper. 1995. Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature* 373:255–257.
40. Wen, L., D. F. Barber, W. Pao, F. S. Wong, M. J. Owen, and A. Hayday. 1998. Primary gamma delta cell clones can be defined phenotypically and functionally as Th1/Th2 cells and illustrate the association of CD4 with Th2 differentiation. *J. Immunol.* 160:1965–1974.
41. Jaffar, Z. H., L. Stanciu, A. Pandit, J. Lordan, S. T. Holgate, and K. Roberts. 1999. Essential role for both CD80 and CD86 costimulation, but not CD40 interactions, in allergen-induced Th2 cytokine production from asthmatic bronchial tissue: role for alphabeta, but not gammadelta, T cells. *J. Immunol.* 163:6283–6291.
42. Boismenu, R., L. Feng, Y. Y. Xia, J. C. Chang, and W. L. Havran. 1996. Chemokine expression by intraepithelial gamma delta T cells: implications for the recruitment of inflammatory cells to damaged epithelia. *J. Immunol.* 157:985–992.
43. Krouwels, F. H., R. E. Nocker, M. Snoek, R. Lutter, J. S. van der Zee, F. R. Weller, H. M. Jansen, and T. A. Out. 1997. Immunocytochemical and flow cytometric detection of intracellular IL-4, IL-5 and IFN-gamma: applications using blood- and airway-derived cells. *J. Immunol. Methods* 203:89–101.
44. Winterrowd, G. E., and J. E. Chin. 1999. Flow cytometric detection of antigen-specific cytokine responses in lung T cells in a murine model of pulmonary inflammation. *J. Immunol. Methods* 226:105–118.