CD4+ Invariant T-Cell–Receptor+ Natural Killer T Cells in Bronchial Asthma

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ABSTRACT

BACKGROUND

Bronchial asthma is associated with an inflammatory process that is characterized by the presence in the airways of large numbers of CD4+ T cells producing interleukin-4 and interleukin-13. However, the CD4 antigen is expressed not only by class II major histocompatibility complex (MHC)–restricted CD4+ T cells, but also by a newly identified subgroup of T cells, CD1d-restricted natural killer T cells. These cells express a conserved (invariant) T-cell receptor and have a potent immunoregulatory function. Because mouse models of allergic asthma indicate that natural killer T cells are required for the development of allergen-induced airway hyperreactivity, we hypothesized that natural killer T cells play an important role in human asthma.

METHODS

We used CD1d-tetramers, antibodies specific for natural killer T cells, as well as reverse-transcriptase–polymerase-chain-reaction analysis of the invariant T-cell receptor of natural killer T cells to assess the frequency and distribution of natural killer T cells in the lungs and in the circulating blood of 14 patients with asthma.

RESULTS

About 60 percent of the pulmonary CD4+CD3+ cells in patients with moderate-to-severe persistent asthma were not class II MHC–restricted CD4+ T cells but, rather, natural killer T cells. The natural killer T cells expressed an invariant T-cell receptor and produced type 2 helper cytokines. In contrast, the CD4+ T cells found in the lungs of patients with sarcoidosis were conventional CD4+CD3+ T cells, not natural killer T cells.

CONCLUSIONS

Together with studies in mice indicating a requirement for natural killer T cells in the development of allergen-induced airway hyperreactivity, our results strongly suggest that CD4+ natural killer T cells play a prominent pathogenic role in human asthma.

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ASTHMA IS CHARACTERIZED BY AIRWAY inflammation dominated by the presence of eosinophils and CD4+ T lymphocytes.[1,2] The pulmonary CD4+ cells in patients with asthma produce predominantly the type 2 helper (Th2) cytokines interleukin-4, interleukin-5, and interleukin-13, which play essential roles in asthma by enhancing the growth, differentiation, and recruitment of eosinophils, basophils, mast cells, and IgE-producing B cells and by directly inducing airway hyperreactivity,[3-5] a cardinal feature of asthma. Thus, class II major histocompatibility complex (MHC)–restricted CD4+ Th2 T cells, which have been detected in the airways of virtually all patients with asthma, are thought to play an essential role in the pathogenesis of bronchial asthma.[6,7]

The CD4 cell surface marker is expressed not only by conventional class II–restricted CD4+ T cells but also by natural killer T cells, a newly described, unique subgroup of lymphocytes that express features of both classic T cells and natural killer cells. In humans, natural killer T cells express CD4, CD8 (a small subgroup), or neither (i.e., negative for both CD4 and CD8 surface markers, also called double-negative cells). Many natural killer T cells express a highly restricted repertoire of T-cell receptors consisting of $V\alpha14$-$J\alpha18$ (in mice) and $V\alpha24$-$J\alpha18$ (in humans) and are called invariant T-cell receptor–positive natural killer T cells (invariant natural killer T cells).[8] This T-cell receptor endows invariant natural killer T cells with the unique property of responding to glycolipid antigens, rather than peptide antigens presented by the nonpolymorphic class I MHC–like protein CD1d, expressed on antigen-presenting cells. Furthermore, on activation, invariant natural killer T cells rapidly produce large quantities of both type 1 helper (Th1)–biased (interferon-γ) and Th2-biased cytokines (interleukin-4), which enhance the function of dendritic cells, natural killer cells, and B cells, as well as the function of conventional CD4+ and CD8+ T cells.[9] This rapid production of cytokines by invariant natural killer T cells is a manifestation of innate-like immunity and provides invariant natural killer T cells with the capacity to link innate and adaptive immune responses and critically regulate adaptive immunity and a host of inflammatory diseases.[10-16] However, the role of invariant natural killer T cells in humans is not completely understood. To investigate whether these invariant natural killer T cells have an important role in human asthma, we studied the frequency and distribution of CD1d-restricted invariant natural killer T cells in the lungs and peripheral blood of patients with persistent asthma.

METHODS

STUDY POPULATION

The panel on medical human subjects of Stanford University, the committee on clinical investigation of Children's Hospital Boston, and the internal review board of the Karolinska Institute in Stockholm approved the study, and written informed consent was obtained from the 25 patients enrolled. Of these, the 14 patients who had asthma were lifelong nonsmokers who had received a diagnosis of moderate-to-severe persistent asthma.

STUDY PROCEDURES

All study patients and healthy controls underwent blood drawing and fiberoptic bronchoscopy. Before transoral fiberoptic bronchoscopy (BF-XT 20 or BF-IT 30 bronchoscope, Olympus) was performed, spirometry was performed (before and after the administration of albuterol) with the use of equipment and procedures that met the guidelines of the American Thoracic Society.[17] Patients and controls were required to have a baseline forced expiratory volume in one second (FEV_1) of more than 40 percent of the predicted value. For entry into the study, patients with asthma were required to have variable airflow obstruction as documented by a variability of more than 30 percent during serial recordings of the peak expiratory flow rate[2] and had to demonstrate both an increase of 250 ml and an increase of 12.5 percent in FEV_1 after treatment with inhaled albuterol. Transoral fiberoptic bronchoscopy was performed as previously described.[17] Peripheral-blood mononuclear cells were obtained from whole blood from donors and was processed as described in the Supplementary Appendix (available with the full text of this article at www.nejm.org).

STATISTICAL ANALYSIS

The statistical analysis was performed with In-Stat software, version 3.05 (GraphPad). The data are reported as means ±SD. Comparisons among the four groups included in the study — patients

with asthma treated with corticosteroids, those with asthma not treated with corticosteroids, those with sarcoidosis, and control subjects — were performed with the Kruskal–Wallis test, with the use of Dunn's method for multiple comparisons. P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

We studied 14 patients with moderate-to-severe persistent asthma, 6 controls, and 5 patients with sarcoidosis, a respiratory inflammatory disease in which large numbers of CD4+ Th1 cells are present in the lungs[18,19] (Table 1). No patient who had asthma had had an exacerbation of the disease or had received oral corticosteroid therapy or theophylline within the three months before entry into the study. The four patients with asthma who had not received inhaled corticosteroids within three months or longer before entry into the study had a mean predicted FEV_1 of 71 percent, indicating clinically significant asthma (Table 1). Patients with atopic asthma had higher serum total IgE levels (mean, 361 IU per milliliter) than both patients who did not have atopic asthma (mean, 53 IU per milliliter) and controls (mean, 21 IU per milliliter). Although patients with asthma who had received corticosteroids had a higher mean serum IgE level (mean, 331 IU per milliliter) than those with asthma not treated with corticosteroids (mean, 118 IU per milliliter), this difference was not significant.

The six control subjects were all asymptomatic volunteers with normal lung function without evidence of variable airflow obstruction, according to serial peak-flow measures. The five patients with sarcoidosis had stage II disease (lymphadenopathy and parenchymal lung findings) with bilateral hilar lymphadenopathy with evidence of reticulonodular shadowing or pulmonary infiltrates on high-resolution computed tomography (thin sections, 1 mm thick) of the lung. No patient with sarcoidosis had a history of erythema nodosum. All five were white (race was determined by physicians in this study), and all had noncaseating granulomas on transbronchial biopsy with negative fungal and acid-fast smears and cultures. The average duration of disease in these patients was six months. None had received treatment with oral or inhaled corticosteroids or other immunosuppressive agents.

BRONCHOALVEOLAR LAVAGE FINDINGS

When specimens of bronchoalveolar-lavage fluid obtained from all study patients and controls were examined for the presence of CD4+ cells, CD8+ cells, and invariant natural killer T cells, we expected and found a higher total cell count in specimens from patients with asthma or sarcoidosis than in those from controls (Table 2). We also noted an increase in the proportion of lymphocytes in patients with asthma (13 percent) and in patients with sarcoidosis (21 percent), as compared with controls (7 percent), but these differences did not reach significance. In both the asthma and sarcoidosis groups, the majority of lymphocytes were CD4+. We then examined the bronchoalveolar-lavage fluid for the presence of invariant natural killer T cells using CD1d tetramers loaded with α-galactosylceramide, which specifically bind to the invariant T-cell receptor of invariant natural killer T cells,[20] and with the monoclonal antibody 6B11, which specifically recognizes the CDR3 region of the Vα24-Jα18 T-cell receptor of human invariant natural killer T cells.[21] Both reagents stained a large number of cells in the bronchoalveolar-lavage fluid obtained from patients with asthma, indicating that invariant natural killer T cells were present in the lungs of these patients (Fig. 1 in the Supplementary Appendix). By contrast, virtually no invariant natural killer T cells were detectable in the bronchoalveolar-lavage fluid from either controls or patients with sarcoidosis.

Because invariant natural killer T cells can express the CD4 cell surface marker, and because large numbers of CD4+ cells are known to be present in the lungs of patients with asthma, we measured the fraction of the CD4+ T cells in bronchoalveolar-lavage fluid of patients with asthma that were invariant natural killer T cells. Surprisingly, we found that a large fraction of these CD4+ T cells were invariant natural killer T cells. In patients with asthma, 45 to 86 percent (mean, 63 percent) of the CD4+ cells expressed the invariant T-cell receptor Vα24, as determined with the use of tetramer staining (Fig. 1A, and Table 2 in the Supplementary Appendix), whereas in patients with sarcoidosis (Fig. 1B) and controls (Fig. 1C), less than 1 percent of the CD4+ cells expressed the invariant T-cell receptor Vα24.

Similar results were obtained with the use of direct immunofluorescence and confocal laser scanning microscopy of biopsy specimens from

Table 1. Characteristics of Patients with Asthma and Results of Radioallergosorbent Testing and Studies of Lung Function.*

Group	Patient No.	Age (yr)	Sex	Duration of Asthma (yr)	Medications Received	IgE (IU/ml)	Allergic Status	FEV₁ (% of predicted)	FEV₁:FVC
Patients with asthma treated with corticosteroids	1	56	M	30	Albuterol, fluticasone propionate	248	Bermuda grass, rye grass, timothy grass, Alternaria mold, cladosporium mold, cat, dust, egg white, milk	55	0.57
	2	26	F	16	Albuterol, fluticasone propionate, salmeterol		Not tested	87	0.74
	3	60	M	40	Albuterol, fluticasone propionate, salmeterol	581	Bermuda grass, rye grass, timothy grass, cladosporium mold, cockroach, dust, corn	92	0.70
	4	36	M	10	Albuterol, fluticasone propionate, salmeterol	190	Olive tree, cockroach, cat, dog, dust	72	0.70
	5	54	F	14	Albuterol, fluticasone propionate, salmeterol, montelukast	79	Negative	109	0.71
	6	56	M	10	Albuterol, fluticasone propionate, salmeterol	41	Negative	87	0.75
	7	52	F	8	Albuterol, fluticasone propionate, salmeterol, montelukast	301	Olive tree, Bermuda grass, rye grass, timothy grass	100	0.79
	8	24	M	24	Albuterol, fluticasone propionate, salmeterol	968	Cat, pollen	85	0.71
	9	34	F	14	Albuterol, fluticasone propionate, salmeterol	529	Cat, pollen, rye grass	56	0.57
	10	45	F	35	Albuterol, fluticasone propionate	44	Negative	100	0.75
Mean value		44		20		331†		84	0.70‡
Patients with asthma not treated with corticosteroids	11	32	F	1	Albuterol	7	Cockroach	75	0.71
	12	43	F	5	Albuterol	40	Negative	85	0.76
	13	31	M	28	Albuterol	305	Cat dander, dog dander, dust mite	73	0.70

14	36	M	30	Albuterol	121	Alder tree, oak tree, olive tree, Bermuda grass, rye grass, timothy grass, western ragweed, Alternaria mold, cladosporium mold, cat dander, dog dander, dust mite	52	0.67
Mean value	36		16		118		71§	0.71
Controls								
15	67	M	0	None	Not tested		94	0.83
16	25	F	0	None	30	Negative	95	0.84
17	52	F	0	None	Not tested		105	0.86
18	27	F	0	None	21	Bermuda grass, rye grass, timothy grass	112	0.80
19	30	F	0	None	20	Negative	102	0.70
20	21	M	0	None	14	Negative	99	0.77
Mean value	37		0		21†		101¶	0.80
Patients with sarcoidosis								
21	55	M	0.5	None	—		132	0.85
22	53	F	0.5	None	—		102	0.78
23	37	M	0.5	None	—		98	0.84
24	41	M	0.5	None	—		96	0.84
25	56	M	0.75	None	—		132	0.85
Mean value	48		0.5‖				112§	0.83‡§

* Patients with asthma took inhaled albuterol. Patients 1 through 10, who had asthma treated with corticosteroids, took inhaled fluticasone propionate. Patients 2 through 9 also took salmeterol, and Patients 5 and 7 also took oral montelukast. Patients 10 through 14, who had asthma, had not received corticosteroid therapy within the three months before entering the study. None of the patients or controls had received monoclonal anti-IgE therapy, and none had an exacerbation of asthma or had received oral corticosteroid therapy or theophylline within the three months, before entering the study. Patients with sarcoidosis had never received corticosteroid therapy. Patient 2 and Control Subject 20 were black; Patients 3, 4, and 10 were Hispanic; and the remaining patients and controls were white. Race was determined by physicians. Patients and controls were considered allergic if they had elevated total IgE, allergen-specific IgE to at least one of a panel of common allergens, and a history of bronchospasm after allergen exposure. Patients with sarcoidosis and controls served as control populations. None of the study subjects had other concurrent illnesses or were pregnant or lactating. FEV_1 denotes forced expiratory volume in one second, and FVC forced vital capacity.

† P<0.01 by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between patients with asthma treated with corticosteroids and controls (P<0.05).

‡ P<0.01 by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between patients with sarcoidosis and those with asthma treated with corticosteroids (P<0.05).

§ P<0.01 by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between patients with sarcoidosis and those with asthma who were not treated with corticosteroids (P<0.05).

¶ P<0.01 by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between controls and patients with asthma who were not treated with corticosteroids (P<0.05).

‖ P<0.05 by the Mann–Whitney test, for the comparison between the means in the groups of patients with asthma and the group of patients with sarcoidosis.

Table 2. Analysis of Cells in the Bronchoalveolar-Lavage Fluid.*

Group	Patient No.	Cell Count									
		Total Cell Count	Macrophages	Neutrophils	Eosinophils	Lymphocytes	CD3+ Cells	CD4+ Cells	CD8+ Cells	Invariant NKT Cells % of CD3+ cells	Invariant NKT Cells % of CD4+ cells
		$\times 10^{-5}/ml$	*percent*								
Patients with asthma treated with corticosteroids	1	45	84	2	<1	14	97	60	36	85	71
	2	62	74	0	0	26	93	38	60	58	45
	3	72	90	2	<1	12	98	68	28	82	52
	4	62	91	0	0	9	94	70	25	65	58
	5	87	92	0	0	8	98	40	53	—	—
	6	120	81	5	0	14	95	85	9	—	—
	7	38	86	0	0	14	97	64	32	—	—
	8	101	87	0	1	11	93	37	46	72	70
	9	61	90	1	1	8	96	55	35	86	65
	10	68	85	1	3	11	96	58	36	77	61
Mean		72†	86	1	1	13	96‡	61	35	73§	60†§
Patients with asthma not treated with corticosteroids	11	72	72	1	2	25	93	50	38	78	86
	12	119	85	3	2	9	90	67	22	75	64
	13	99	86	4	2	8	92	37	51	73	61
	14	12	77	3	16	4	87	43	43	63	58
Mean		76	80	3	6	12	91†	49	39	72¶	67¶‖

Healthy controls	15	25	95	2	0	3	93	50	47	0.8	0.5
	16	52	94	3	1	2	87	39	41	0.4	0.4
	17	31	89	5	0	6	88	32	56	1.1	0.6
	18	6	83	7	0	9	91	57	31	0.9	0.7
	19	78	96	0	0	4	90	60	28	1.2	0.5
	20	71	85	0		15	97	16	77	1.3	0.9
Mean		53**	90	3	0	7	91	43	44	0.9§¶	0.6§¶
Patients with sarcoidosis	21	142	48	2	0	40	94	68	22	1.3	0.1
	22	186	91	0	0	9	93	77	17	1.4	0.7
	23	240	65	4	1	30	88	138	0.5†	1.6	0.5
	24	127	94	1	0	4	87	63	35	1.2	0.4
	25	155	80	0	0	22	94	68	22	1.4	0.8
Mean		170†**	76	1	0	21	91	72	22	1.38	0.5†‖

* Differential cell counts for macrophages, neutrophils, eosinophils, and lymphocytes are expressed as percentages of the total cell count. The CD3+, CD4+, and CD8+ cell counts are given as percentages of the lymphocyte count. NK denotes natural killer. Because the bronchoalveolar-lavage fluid samples from Patients 5, 6, and 7 were stained with CD1d tetramers or 6B11 monoclonal antibody, but not with anti-CD3 or anti-CD4, we could not calculate the percentages of CD3 or CD4 cells that were invariant natural killer T cells for these patients. Dashes indicate that data were not available.

† $P<0.01$ by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between patients with asthma who were treated with corticosteroids and patients with sarcoidosis ($P<0.05$).
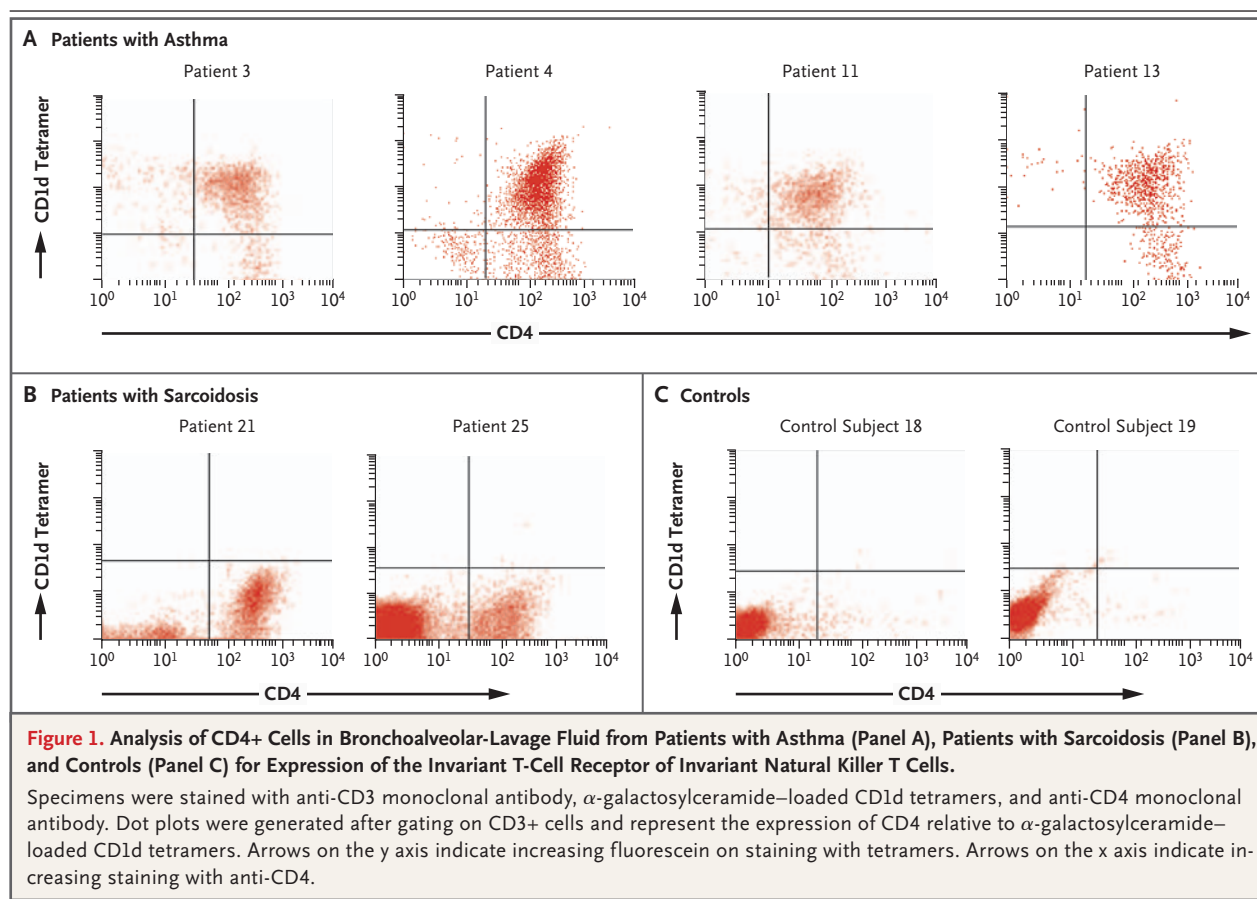
‡ $P<0.05$ by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between patients with asthma who were treated with corticosteroids and those not treated with corticosteroids ($P<0.05$).

§ $P<0.01$ by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between patients with asthma who were treated with corticosteroids and controls ($P<0.05$).

¶ $P<0.01$ by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between controls and patients with asthma who were not treated with corticosteroids ($P<0.05$).

‖ $P<0.01$ by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between patients with sarcoidosis and patients with asthma who were not treated with corticosteroids ($P<0.05$).

** $P<0.01$ by the Kruskal–Wallis test, after testing with Dunn's method for multiple comparisons showed a significant difference between patients with sarcoidosis and controls ($P<0.01$).

Figure 1. Analysis of CD4+ Cells in Bronchoalveolar-Lavage Fluid from Patients with Asthma (Panel A), Patients with Sarcoidosis (Panel B), and Controls (Panel C) for Expression of the Invariant T-Cell Receptor of Invariant Natural Killer T Cells.

Specimens were stained with anti-CD3 monoclonal antibody, α-galactosylceramide–loaded CD1d tetramers, and anti-CD4 monoclonal antibody. Dot plots were generated after gating on CD3+ cells and represent the expression of CD4 relative to α-galactosylceramide–loaded CD1d tetramers. Arrows on the y axis indicate increasing fluorescein on staining with tetramers. Arrows on the x axis indicate increasing staining with anti-CD4.

patients with asthma (Fig. 2A through 2D). A photomicrograph of one biopsy specimen (Fig. 2A) shows the typical features of bronchial asthma — thickening of the basement membrane (lamina reticularis), epithelial disruption, and the presence of a mononuclear cell infiltrate, including invariant natural killer T cells, in the submucosa (lamina propria). In findings on confocal laser microscopy (Fig. 2B), nearly all the lymphocytes in the lamina propria express both CD4 and the invariant T cell receptor Vα24; in contrast, in patients with sarcoidosis, the lymphocytes express CD4 but not Vα24 and therefore are not invariant natural killer T cells (Fig. 2C).

Analysis of the bronchoalveolar-lavage fluid obtained from patients with asthma indicated that 58 to 86 percent (mean, 74 percent) of the CD3+ cells were invariant natural killer T cells (Fig. 2D), whereas in patients with sarcoidosis, less than 2 percent of the CD3+ cells were invariant natural killer T cells (Fig. 2D, and Table 2 in the Supplementary Appendix). The number of invariant natural killer T cells in the lungs of the

14 patients with asthma did not appear to be significantly reduced with inhaled corticosteroid therapy: 10 of these patients had been treated with potent inhaled corticosteroids for six months or longer before they underwent bronchoscopy, yet the majority of the pulmonary CD3+ cells from the patients (Patients 1, 2, 3, 4, 8, 9, and 10) (Fig. 2D) expressed the invariant T-cell receptor of invariant natural killer T cells, a finding similar to that observed in patients who had not been treated with corticosteroids (Patients 11, 12, 13, and 14).

To confirm the results of our study performed with the use of CD1d tetramers and the natural killer T-cell–specific antibody, we also performed semiquantitative reverse-transcriptase–polymerase-chain-reaction analysis. This molecular analysis demonstrated a high expression of the messenger RNA (mRNA) for the invariant T-cell receptor of invariant natural killer T cells in the lungs of patients with asthma. The mRNA for Vα24 and Vβ11 (the invariant T-cell receptor of natural killer T cells), but not Vα23 (an irrelevant T-cell receptor), was strongly expressed in

Figure 2. CD4+ Invariant Natural Killer T (NKT) Cells in the Airways of Patients with Asthma, but Not Patients with Sarcoidosis.

The left-hand portion of Panel A shows an endobronchial-biopsy specimen obtained from a patient with asthma with typical features of chronic asthma, including thickening of the basement membrane (lamina reticularis) (arrowhead), epithelial disruption, and cell infiltrates in the submucosa and lamina propria. On the right-hand side, a section from the same specimen shows staining of cells immediately beneath the lamina reticularis with fluorescein isothiocyanate–conjugated (FITC) antibody (monoclonal antibody 6B11) against the invariant natural killer T-cell receptor (arrow). Laser confocal images of bronchial-biopsy specimens from a patient with asthma are shown in Panel B and from a patient with sarcoidosis in Panel C. The CD4+ cells from the patient with bronchial asthma are invariant NKT cells, but those from the patient with sarcoidosis are not. A lung-biopsy specimen was stained with phycoerythrin-conjugated CD4 (red), and FITC-conjugated 6B11 monoclonal antibody (blue). The overlay results (pink) indicate that almost none of the CD4+ lymphocytes from the patient with sarcoidosis but nearly all of the CD4+ infiltrating lymphocytes from the patient with asthma coexpressed the invariant T-cell receptor. Panel D shows the percentage of CD3+ cells that are invariant NKT cells in bronchoalveolar-lavage fluid from 11 patients with asthma and 5 patients with sarcoidosis. Patients 11, 12, 13, and 14 did not receive corticosteroids. Cells were stained with anti-CD3 and α-galactosylceramide–loaded CD1d tetramers. The bars represent the percentages of CD3+ cells that are positive or negative for CD1d tetramers in each of the four patients.

cells from the bronchoalveolar-lavage fluid from patients with asthma (Fig. 3), but not in those from patients with sarcoidosis or controls. Together, these studies conducted with several different approaches indicate that CD4+ invariant natural killer T cells are virtually absent from the lungs of controls and patients with sarcoidosis but are present in high numbers in the lungs of patients with asthma.

Although the invariant natural killer T cells in the lungs of patients with asthma were distinct from conventional class II–restricted CD4+ T cells in expressing an invariant T-cell receptor, the invariant natural killer T cells were similar to CD4+ Th2 cells in producing interleukin-4 and interleukin-13. We found that the invariant natural killer T cells in the lungs of patients with asthma produced both interleukin-4 and interleukin-13 but little interferon-γ on intracellular cytokine staining after activation with phorbol myristyl acetate and ionomycin (Fig. 4A) or by
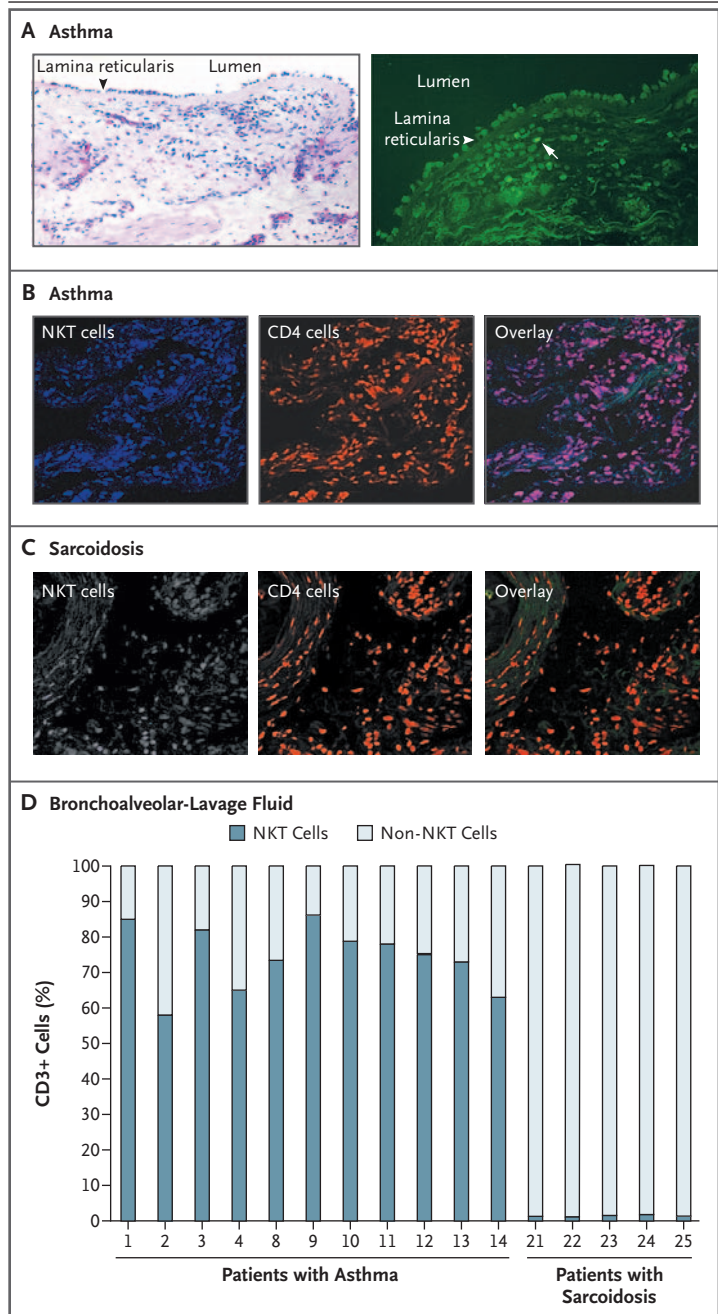
measurement of cytokines in supernatants after activation with α-galactosylceramide, which specifically activates invariant natural killer T cells (Fig. 4B). In contrast, invariant natural killer T cells in the peripheral blood of all the patients with asthma or sarcoidosis and the controls produced all three cytokines (Fig. 4C). Furthermore, in the bronchoalveolar-lavage fluid of patients with asthma, the vast majority (>95 percent) of the invariant natural killer T cells coexpressed CD4+ (Fig.
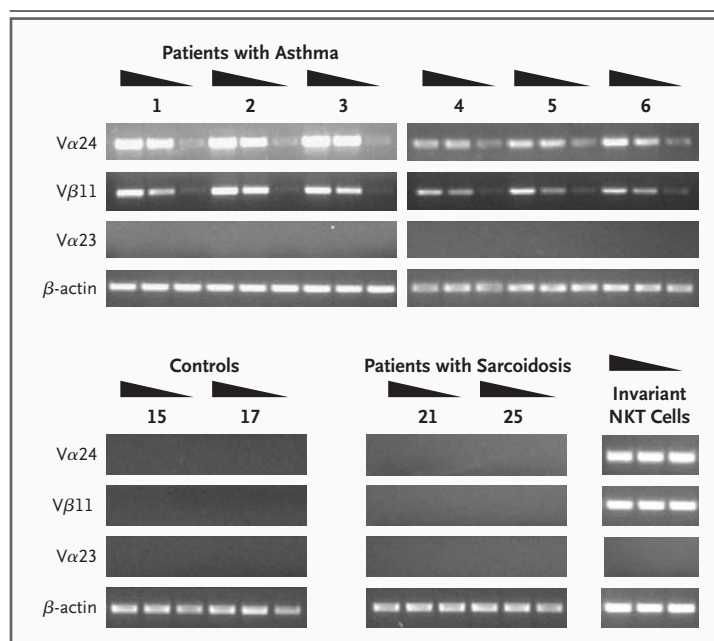
Figure 3. Messenger RNA for the Invariant T-Cell Receptor of Invariant Natural Killer T (NKT) Cells Expressed in Cells Obtained by Bronchoalveolar-Lavage from Patients with Asthma.

Cells from Patients 1 through 6 with asthma, Patients 21 and 25 with sarcoidosis, and two controls (Subjects 15 and 17) were analyzed for the expression of Vα24, Vβ11 (invariant T-cell receptor), and Vα23 (irrelevant T-cell receptor) by reverse-transcriptase polymerase chain reaction (as described in the Supplementary Appendix). For each patient or control, expression was quantitated by amplification with 35, 30, and 25 cycles (loaded on gel left to right for each patient or control, as indicated by the slope of the triangle). To assess RNA loading, β-actin was measured in the same subjects. Messenger RNA from purified invariant NKT cells was used as a standard for expression of Vα24, Vα23, and Vβ11.

Figure 4 (facing page). **Expression of Interleukin-4, Interleukin-13, and Interferon-γ by Invariant Natural Killer (NKT) T Cells in Bronchoalveolar-Lavage Fluid and Peripheral Blood.**

Cells from bronchoalveolar-lavage fluid (Panel A) and purified invariant NKT cells isolated from peripheral blood (Panel C) were stimulated with phorbol myristyl acetate and ionomycin and stained for intracellular cytokines, as described in the Methods section. The open histograms represent the expression of cytokines by invariant NKT cells; the solid histograms depict staining with isotype control antibody. Panel B shows the production of interleukin-4, interleukin-13, and interferon-γ by bronchoalveolar lavage from patients with asthma after culture with α-galactosylceramide or vehicle control. Supernatants were removed after 48 hours and analyzed by enzyme-linked immunosorbent assay. Panel D shows the percentage of all invariant NKT cells in the bronchoalveolar-lavage fluid from six patients with asthma (Panel D) and in peripheral blood from patients with asthma, patients with sarcoidosis, and controls (Panel E) that expressed CD4, CD8, or neither. The bars represent the percentage of invariant NKT cells that are CD4+CD8+ or CD4–CD8– (double negative) in each patient or control.

with sarcoidosis. We confirmed previous work[6,7] showing that T cells in the lungs of patients with asthma expressed the CD4 cell surface marker and produced Th2 cytokines, interleukin-4 and interleukin-13, but not interferon-γ — that is, that these T cells have the typical cytokine profile of conventional CD4+ Th2 lymphocytes. However, we showed that a great proportion (63 percent) of the pulmonary CD4+ T cells in patients with moderate-to-severe persistent asthma (and 73 percent of the CD3+ cells) expressed an invariant T-cell receptor and thus are invariant natural killer T cells, rather than conventional Th2 lymphocytes. The profusion of pulmonary invariant natural killer T cells in patients with asthma that we detected is surprising, but this finding mirrors those in mouse models of allergic asthma showing an essential role for invariant natural killer T cells in the development of allergen-induced airway hyperreactivity.[15,16] Moreover, it is surprising that invariant natural killer T cells are present in the lungs of patients with asthma but not in the lungs of patients with sarcoidosis, a multisystem disorder predominantly involving the lungs. Both patients with sarcoidosis and those with asthma have large numbers of CD4+ T cells in their lungs, but in patients with asthma the T cells secrete interleukin-4 and interleukin-13, whereas in

4D), whereas in the peripheral blood of the patients with sarcoidosis and controls, only about 40 percent of the invariant natural killer T cells were CD4+ cells (approximately 50 percent of the invariant natural killer T cells were negative for both CD4 and CD8, and approximately 3 percent were CD8+) (Fig. 4E). These results suggest that one subgroup of invariant natural killer T cells (those producing Th2 cytokines and expressing CD4) is selectively recruited or expanded in the lungs of patients with bronchial asthma but not in the lungs of patients with sarcoidosis.
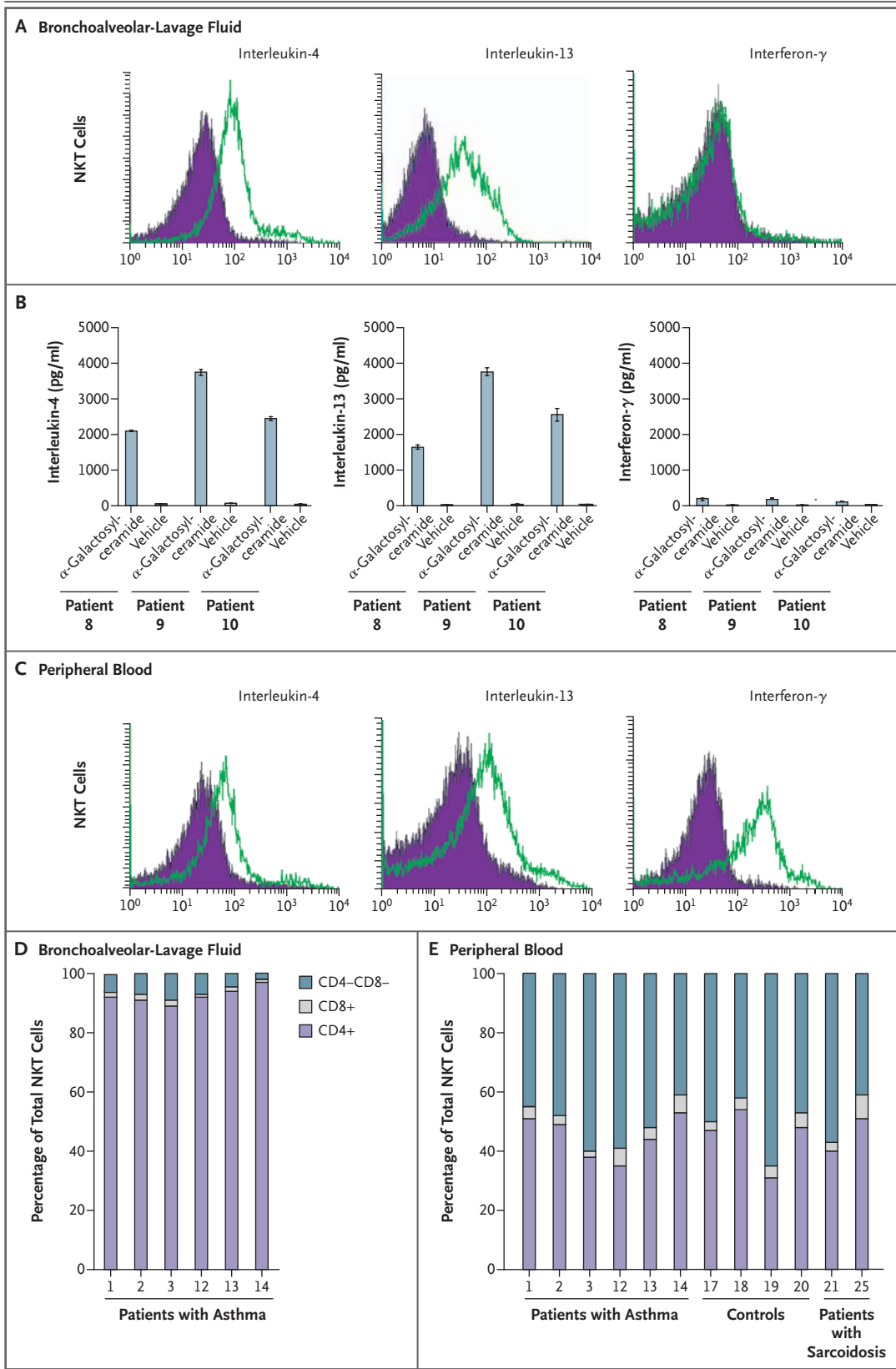
DISCUSSION

Our studies show that CD4+ and CD3+ invariant natural killer T cells are abundant in the lungs of patients with chronic asthma but are virtually absent from the lungs of controls and patients

patients with sarcoidosis the T cells secrete interferon-γ rather than interleukin-4 and interleukin-13.[22,23]

The large number of invariant natural killer T cells in the lungs of patients with asthma is striking, especially given the fact that these cells constitute less than 0.1 percent of the mononuclear cells and less than 1 percent of the CD4+ T cells in the peripheral blood.[24] In addition, our finding that more than 90 percent of the invariant natural killer T cells in the lungs of patients with asthma are CD4+ cells, whereas only about 50 percent of the invariant natural killer T cells in the peripheral blood are CD4+ cells, suggests that a subgroup of invariant natural killer T cells is recruited and enriched in the lung, leading to levels in the lung that are 100 times the levels in the peripheral blood. The preferential recruitment of invariant natural killer T cells may be related to a differential expression of chemokine receptors on the subgroup of CD4+ cells that are invariant natural killer T cells — a subgroup thought preferentially to produce interleukin-4 and interleukin-13.[25-28] Accordingly, our study indicates that the immunology of asthma must be studied not by the examination of peripheral blood but, rather, by the evaluation of cells from within the lung. This principle may also hold true for other diseases in which invariant natural killer T cells have been reported to play an important role.

To identify invariant natural killer T cells, we used CD1d tetramers loaded with α-galactosylceramide, monoclonal antibody 6B11, or both, currently considered the most sensitive and specific reagents for detecting invariant natural killer T cells. We found that other reagents, such as antibody to the T-cell receptors Vα24 and Vβ11, although effective in identifying resting invariant natural killer T cells in peripheral blood, were less sensitive than CD1d tetramers and monoclonal antibody 6B11 in detecting invariant natural killer T cells in bronchoalveolar-lavage fluid. This finding might be due to the fact that the invariant natural killer T cells in the lungs of patients with asthma are partially activated, even in stable asthma, and that the T-cell–receptor expression on invariant natural killer T cells is greatly down-regulated after the activation of invariant natural killer T cells.[29] However, levels of Vα24 and Vβ11 mRNA were highly expressed in cells in bronchoalveolar-lavage fluid (Fig. 3), a finding consistent with the idea that T-cell receptor down-

regulation reduces the sensitivity of detection of invariant natural killer T cells with anti-Vα24 and anti-Vβ11 antibody. We cannot exclude the possibility that even with the use of CD1d tetramers, monoclonal antibody 6B11, or both to identify invariant natural killer T cells in bronchoalveolar-lavage fluid, the frequency of invariant natural killer T cells in the lungs of patients with asthma might be underestimated because of T-cell receptor down-regulation.

CD4+ invariant natural killer T cells in the lungs of patients with asthma express an invariant T-cell receptor that recognizes glycolipid antigens that are now being defined.[30] These antigens appear to be highly conserved in mice and humans and include the synthetic glycolipid α-galactosylceramide, the self-glycolipid isoglobotrihexosylceramide (iGb3),[31,32] bacterial glycosphingolipids,[33,34] and glycolipids from plant pollens.[35] However, we propose that self-glycolipids such as iGb3, which may be exposed in the lungs as a result of pulmonary inflammation or lung injury, can activate invariant natural killer T cells, leading to airway inflammation and asthma. Alternatively, exogenous glycolipids, such as those from inhaled plant pollens, may activate invariant natural killer T cells in the lungs and cause asthma. Identifying the glycolipids recognized by the invariant T-cell receptor of natural killer T cells, and understanding the processes by which glycolipids are generated and activate invariant natural killer T cells, will probably provide important insights into asthma pathogenesis and perhaps reveal a host of new pathways amenable to new treatments specifically for asthma.

In summary, we found that a large fraction of the CD4+ T cells in the lungs of patients with asthma, but not in the lungs of patients with sarcoidosis, express the invariant T-cell receptor of invariant natural killer T cells, a newly described subgroup of T cells with immunoregulatory function. Together with studies in mice indicating the requirement of invariant natural killer T cells for the development of allergen-induced airway hyperreactivity, our results strongly suggest that invariant natural killer T cells in asthma represent a new paradigm in which CD4+ invariant natural killer T cells, in concert with conventional CD4+ T cells, produce interleukin-4 and interleukin-13, driving the development of inflammation in bronchial asthma. If invariant natural killer T cells do indeed play a prominent

role in the pathogenesis of asthma, therapies for asthma that target pulmonary invariant natural killer T cells may be highly effective.

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