

ORIGINAL ARTICLE

Invariant Natural Killer T Cells in Asthma and Chronic Obstructive Pulmonary Disease

Pandurangan Vijayanand, M.D., Grégory Seumois, M.Sc.,
Chris Pickard, Ph.D., Robert M. Powell, Ph.D., Gilbert Angco, B.A.A.,
David Sammut, M.D., Stephan D. Gadola, M.D., Peter S. Friedmann, M.D.,
and Ratko Djukanović, M.D.

ABSTRACT

BACKGROUND

From the Division of Infection, Inflammation, and Repair, University of Southampton, Southampton General Hospital (P.V., G.S., C.P., G.A., D.S., P.S.F., R.D.); and PrimerDesign, Roger Brooke Laboratory, University of Southampton, Southampton General Hospital (R.M.P.) — both in Southampton, United Kingdom; and University of Bern, Inselspital, Bern, Switzerland (S.D.G.). Address reprint requests to Dr. Vijayanand at the Inflammatory Cell Biology Group, Division of Infection, Inflammation, and Repair, Level F, South Block, Mail Point 810, Southampton General Hospital, Tremona Rd., Southampton SO16 6YD, United Kingdom, or at vijay@soton.ac.uk.

Dr. Vijayanand and Mr. Seumois contributed equally to this article.

N Engl J Med 2007;356:1410-22.
Copyright © 2007 Massachusetts Medical Society.

The number of type 2 helper CD4+ T cells is increased in the airways of persons with asthma. Whether the majority of these cells are class II major-histocompatibility-complex–restricted cells or are among the recently identified CD1d-restricted invariant natural killer T cells is a matter of controversy. We studied the frequency of invariant natural killer T cells in the airways of subjects with mild or moderately severe asthma to investigate the possibility of an association between the number of invariant natural killer T cells in the airway and disease severity. We also studied whether an increased number of these cells is a feature of chronic obstructive pulmonary disease (COPD).

METHODS

We enumerated invariant natural killer T cells by flow cytometry with the use of CD1d tetramers loaded with α -galactosylceramide and antibodies specific to the invariant natural killer T-cell receptor in samples of bronchoalveolar-lavage fluid, induced sputum, and bronchial-biopsy specimens obtained from subjects with mild or moderately severe asthma, subjects with COPD, and healthy control subjects. Real-time polymerase-chain-reaction analysis was performed on bronchoalveolar-lavage cells for evidence of gene expression of the invariant natural killer T-cell receptor.

RESULTS

Fewer than 2% of the T cells obtained from all subjects on airway biopsy, bronchoalveolar lavage, and sputum induction were invariant natural killer T cells, with no significant differences among the three groups of subjects. No expression of messenger RNA for the invariant natural killer T-cell–receptor domains $V\alpha 24$ and $V\beta 11$ was detected in bronchoalveolar-lavage cells from subjects with asthma.

CONCLUSIONS

Invariant natural killer T cells are found in low numbers in the airways of subjects with asthma, subjects with COPD, and controls.

ASTHMA IS CHARACTERIZED BY A SPECIFIC form of airway inflammation that is believed to be orchestrated by CD4⁺ helper T cells of the type 2 (Th2) phenotype in response to allergens presented by mucosal dendritic cells.¹ Th2 CD4⁺ T cells release the cytokines interleukin-4, interleukin-5, and interleukin-13, which play key roles in airway inflammation and remodeling.² The Th2 CD4⁺ T cells have been generally accepted to be class II major-histocompatibility-complex (MHC)–restricted cells.³ This notion has recently been challenged in a study by Akbari and colleagues,⁴ which found that about 60% of the CD4⁺ T cells in the airways of persons with asthma, but not those in the airways of healthy persons, are invariant natural killer T cells, a subgroup of immunoregulatory T lymphocytes restricted by the antigen-presenting molecule CD1d. Their study also showed that bronchoalveolar-lavage cells challenged with α -galactosylceramide, a potent stimulus for invariant natural killer T cells, produce Th2 cytokines, implying a pivotal role in allergic airway inflammation.⁴ There have been two other reports of elevated levels of invariant natural killer T cells in the airways of persons with asthma,^{5,6} although both of them reported lower cell counts than those reported by Akbari et al. More recently, the findings of Akbari et al. have been challenged by Thomas et al.,⁷ who found low numbers of invariant natural killer T cells (0.4 to 2.1%) in persons with asthma not treated with corticosteroids.

We further investigated the role of invariant natural killer T cells in airway disease to determine whether these cells are not only a feature of moderately severe asthma requiring treatment with inhaled corticosteroids, as reported by Akbari et al.,⁴ but are also abundant in mild asthma and chronic obstructive pulmonary disease (COPD). We obtained samples from the airways of subjects with asthma, subjects with COPD, and healthy controls, using bronchoalveolar lavage, sputum induction, and bronchial biopsy. We examined the specimens for invariant natural killer T cells using a combination of flow cytometry and real-time polymerase-chain-reaction (PCR) analysis of gene expression for the $V\alpha 24$ and $V\beta 11$ domains of the T-cell receptor that characterize invariant natural killer T cells.^{8–10}

METHODS

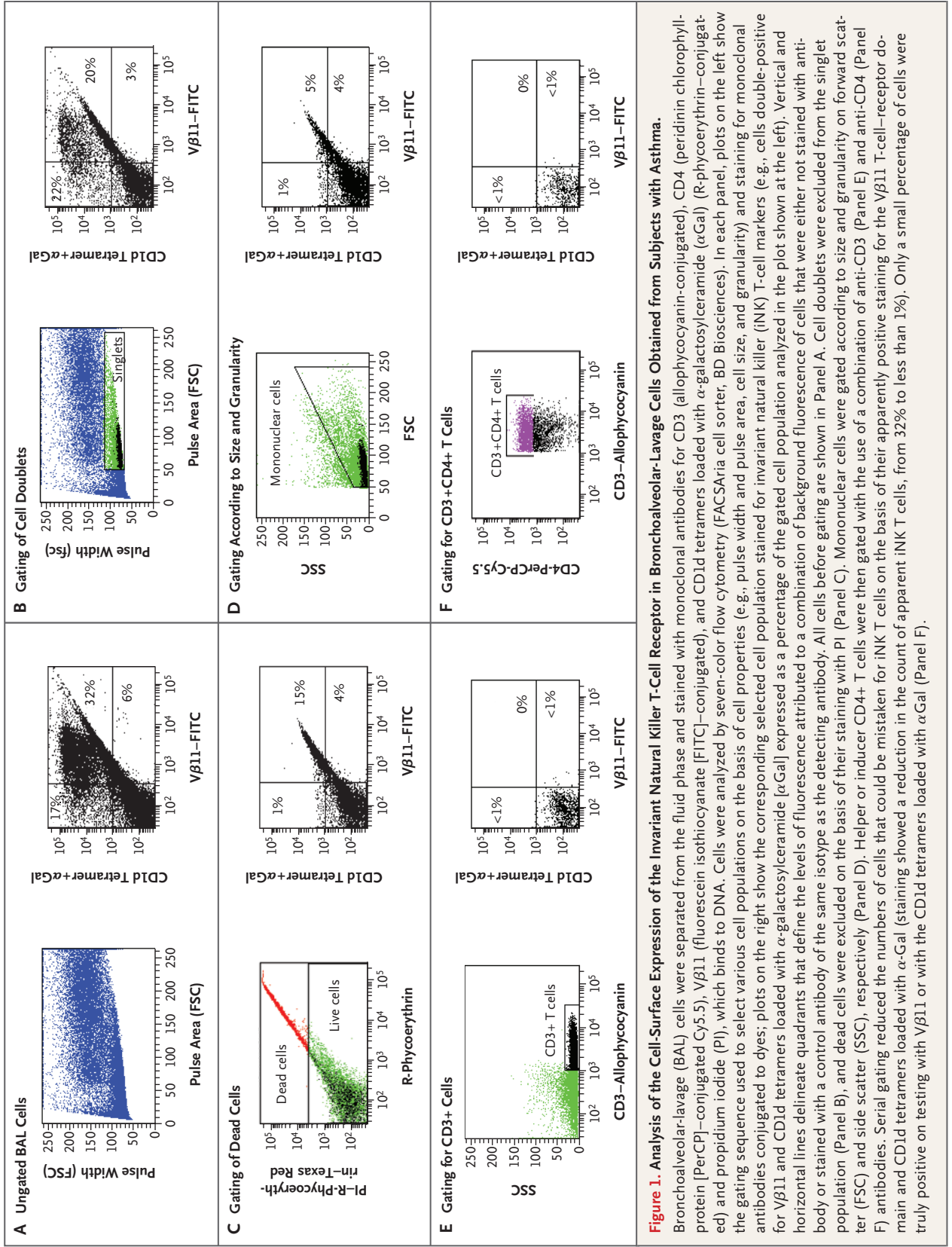
STUDY POPULATION

The study was approved by the ethics committee of the Southampton University Hospitals Trust, and all subjects provided written informed consent. Twenty-four subjects with asthma (8 with mild asthma not treated with inhaled corticosteroids, and 16 with moderately severe asthma treated with inhaled corticosteroids), 10 subjects with COPD meeting established diagnostic criteria,^{11,12} and 12 controls were studied (Table E1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org). All subjects with asthma had had stable symptoms within at least 6 weeks before enrollment. Of these, 22 subjects had atopic asthma, as shown by a positive skin-prick test for a panel of common allergens, and all had bronchial hyperresponsiveness, as demonstrated by the provocative concentration of histamine causing a 20% fall in the forced expiratory volume in 1 second of less than 8 mg per milliliter or peak flow variability of more than 20%. Five of the 10 subjects with COPD were studied during an infectious exacerbation to determine whether there was any contribution by infection to the involvement of invariant natural killer T cells, given the well-established role of these T cells in innate immunity.⁸ All subjects with COPD had disease ranging from stage 0 to stage 3, according to the criteria of the Global Initiative for Chronic Obstructive Lung Disease,¹² and were either current or former heavy smokers; none had a history of asthma or other lung diseases. Exacerbation was defined on clinical and radiologic grounds, according to the guidelines of the British Thoracic Society.¹³

COLLECTION AND PREPARATION OF THE SAMPLES

Sputum induction was performed according to the recommendations of the European Respiratory Society, and the samples were immediately processed with the use of dithioerythritol to separate cells from the fluid phase of sputum.¹⁴ Cell aliquots were immediately processed for flow-cytometric analysis.

Of the 24 subjects with asthma, 18 (5 with mild asthma and 13 with moderately severe asthma) underwent fiberoptic bronchoscopy for either bron-



choalveolar lavage or bronchial biopsy, or both, in accordance with the recommendations of the American Thoracic Society.¹⁵ Cell aliquots from bronchoalveolar-lavage fluid were either subjected to analysis by flow cytometry or placed into lysis buffer for subsequent real-time PCR analysis. Specimens obtained on bronchial biopsy were immediately dispersed with the use of collagenase I (Sigma) reconstituted in RPMI 1640 without L-glutamine (Cambrex) at 1 mg per milliliter for a 1-hour period at 37°C (98.6°F).

PHENOTYPIC ANALYSIS

Cell-surface expression of the invariant natural killer T-cell receptor was detected on flow cytometry with the use of the seven-color FACSAria cell sorter (BD Biosciences) by applying the following monoclonal antibodies in combination with anti-CD3 and anti-CD4 monoclonal antibodies (BD Biosciences): R-phycoerythrin–conjugated anti-V α 24 monoclonal antibody, fluorescein isothiocyanate (FITC)–conjugated anti-V β 11 monoclonal antibody (Immunotech), R-phycoerythrin–conjugated CD1d tetramers loaded with α -galactosylceramide and R-phycoerythrin–conjugated 6B11 monoclonal antibody directed against the complementarity-determining–region 3 of the V α 24-J α 18 T-cell receptor of invariant natural killer T cells (BD Biosciences).

Nonspecific binding of antibodies to Fc γ receptors was blocked with 2 mg per milliliter of polyclonal human IgG (Sigma). Initially, cell doublets, which could contain adherent cells (e.g., macrophages) and dead cells and thus could bind antibodies nonspecifically, were excluded on gating with the use of pulse width and area (Fig. 1, and Fig. E1 in the Supplementary Appendix). Dead cells were further excluded on the basis of their staining with the DNA-binding agent propidium iodide (Fig. 1, and Fig. E2 in the Supplementary Appendix). Large cells and debris were excluded in the forward- and side-scatter plot (Fig. 1). CD3+ T cells were gated positively on the basis of their staining with anti-CD3 antibody conjugated to allophycocyanin, a fluorescent dye that emits light at a wavelength beyond the range of autofluorescent cells (macrophages). As expected, these CD3+ cells were found in a tight group of cells with low granularity (black dots, or cells) (Fig. 1, and Fig. E1 in the Supplementary Appendix). Finally, CD3+CD4+ T cells were gated on the basis of their staining with anti-CD4 antibody. (The gating strategy is described in detail in Fig. E1 through E6 in the Supplementary Appendix.)

QUANTITATIVE, REAL-TIME PCR

We used oligonucleotide probes for real-time PCR of the genes encoding V α 24 (TRAV10, according to the International ImmunoGeneTics [IMGT] information system nomenclature) and V β 11 (TRBV25-1) as well as probes to detect expression of the constant chain of the T-cell receptor (TRBC2 gene) (PrimerDesign). The primers were aligned with other variable regions to confirm that there was no significant homology with any other variable region (for primer detection kits, see the Supplementary Appendix).

RESULTS

BRONCHOALVEOLAR-LAVAGE ANALYSIS

The initial flow-cytometric analyses for invariant natural killer T cells were performed on samples of bronchoalveolar-lavage fluid obtained from subjects with asthma, permitting direct comparisons with previous studies of invariant natural killer T cells in asthma⁴ and sarcoidosis.¹⁶ The analysis of samples obtained from 11 subjects with asthma (3 with mild asthma and 8 with moderately severe asthma) using the V α 24 and V β 11 monoclonal antibodies, which detected all invariant natural killer T cells,^{8–10} showed that invariant natural killer T cells constituted less than 2% of the CD3+ cells and less than 1.5% of the CD3+CD4+ cells (Table 1). Similar counts were obtained when we used CD1d tetramers loaded with α -galactosylceramide¹⁷ or the 6B11 monoclonal antibody (Fig. 1 and 2 and Table 2). Routine total and differential cell counts of bronchoalveolar-lavage cells are shown in Table 3.

To create a positive control for detecting invariant natural killer T cells in bronchoalveolar-lavage cells with the use of flow cytometry, we “spiked” a bronchoalveolar-lavage sample with cells from the invariant natural killer T-cell clone (for validation of the cell clone, see Fig. E7 in the Supplementary Appendix). Using the gating principles outlined in Figure 1 and Figure 2, we detected invariant natural killer T cells in the spiked sample, with the relative numbers (percentages of total CD3+ T cells) corresponding to the numbers of added invariant natural killer T cells (Fig. 3). This confirmed the ability of both the antibodies used and the gating strategy to detect invariant natural killer T cells. Further phenotyping of CD3+CD4+ cells showed these cells to be predominantly activated memory T cells expressing CD69 and CD45RO (Fig. E8 in the Supplementary Appendix).

Table 1. T Cells in Human Airways.*

Sampling Method and Subject No.†	Cell Count			
	CD3+ T Cells % of all cells	CD4+ T Cells % of CD3+ cells	Invariant Natural Killer T Cells % of CD3+ cells	% of CD4+ cells
Subjects with moderately severe asthma treated with inhaled corticosteroids				
Bronchoalveolar lavage				
1	14.4	60.0	0	0
2	0.9	54.0	0.2	0.2
3	0.2	44.3	0	0
4	18.3	56.5	0.1	0.1
5	2.1	64.9	0	0
6	0.3	43.8	0	0
7	22.0	61.0	0	0
8	2.6	36.8	0	0
Bronchial biopsy				
3	4.0	NA	0	NA
4	1.3	NA	0	NA
7	0.2	NA	0	NA
9	7.6	NA	0	NA
10	7.3	NA	0	NA
11	2.3	NA	0	NA
12	4.2	NA	1.3	NA
13	3.3	NA	1.2	NA
Sputum induction				
4	4.6	77.0	0.5	0.5
7	1.6	65.6	0	0
10	4.1	38.6	0.6	0.9
12	5.2	67.5	0.1	0.1
14	0.8	83.3	1.3	1.3
15	0.8	75.0	0	0
16	2.3	58.2	0	0
Subjects with mild asthma not treated with inhaled corticosteroids				
Bronchoalveolar lavage				
17	13.3	55.8	0	0
18	31.0	50.8	0	0
19	0.1	NA	0	NA
Bronchial biopsy				
19	0.2	NA	0	NA
20	5.5	NA	1.7	NA
21	1.1	NA	0.9	NA
Sputum induction				
22	1.7	85.3	0.6	0
23	1.5	70.0	0.1	0.1
24	0.2	71.0	0	0

Table 1. (Continued.)

Sampling Method and Subject No.	Cell Count			
	CD3+ T Cells	CD4+ T Cells	Invariant Natural Killer T Cells	
	% of all cells	% of CD3+ cells	% of CD3+ cells	% of CD4+ cells
Control subjects				
Sputum induction				
25	14.3	80.0	0	0
26	3.4	76.0	0.6	0.8
27	1.5	47.0	0	0
28	1.2	87.5	0.5	0.5
29	1.7	62.0	0.2	0.3
30	7.5	53.2	0	0
31	1.8	63.6	0	0
32	0.7	74.5	0	0
33	1.4	80.4	0	0
34	2.8	58.1	0	0
35	1.3	63.0	0	0
36	3.5	67.8	0.1	0
Subjects with COPD with acute exacerbations				
Sputum induction				
37	0.2	26.8	0	0
38	1.6	76.5	0	0
39	1.5	52.0	0	0.1
40	2.1	78.9	0.1	0.1
41	1.5	40.0	0.1	0.1
Subjects with stable COPD				
Sputum induction				
42	1.5	91.0	0	0
43	1.1	61.7	0	0
44	1.9	65.7	0	0
45	1.0	61.3	0	0
46	1.0	33.0	0	0

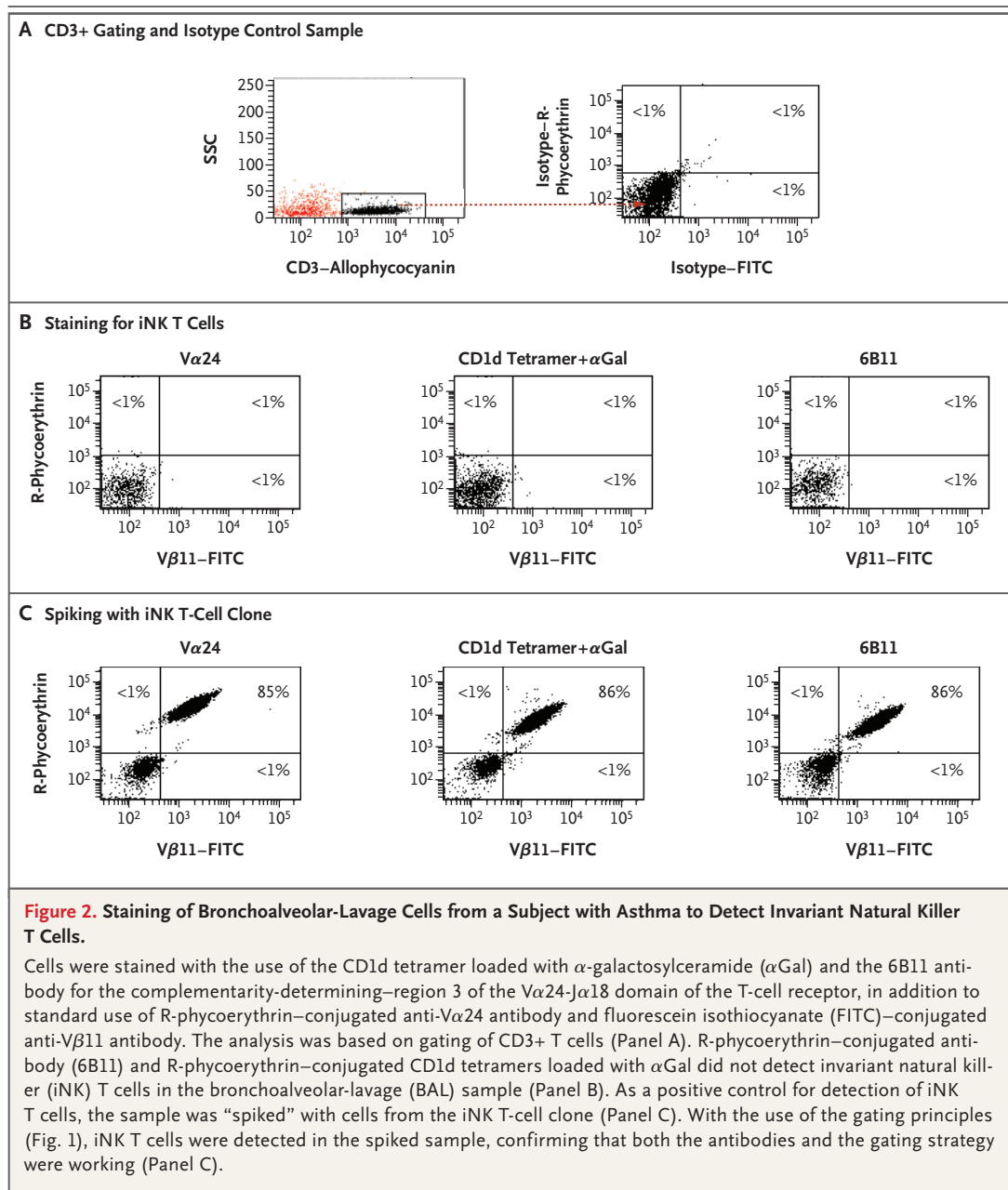
* Percentages of invariant natural killer T cells were identified with the use of double-positive staining for the anti-Vα24 and anti-Vβ11 antibodies, according to the standard method for detecting invariant natural killer T cells. T cells were identified on flow cytometry, by applying the gating strategy (Fig. 1 and 2) and with the use of anti-CD3 antibody. NA denotes not analyzed, and COPD chronic obstructive pulmonary disease.

† There were 16 subjects with moderately severe asthma, 8 with mild asthma, 12 controls, 5 subjects with COPD with acute exacerbations, and 5 with stable COPD. Some subjects underwent sampling by more than one method.

SPUTUM ANALYSIS

The next analysis for invariant natural killer T cells was performed on airway cells obtained by sputum induction,¹⁴ which permitted us to study a larger number of subjects than did the analysis of bronchoalveolar-lavage fluid. This method samples predominantly proximal airways, the sites of significant inflammation in asthma, which could,

conceivably, be different from the distal airways sampled by bronchoalveolar lavage. We also used induced sputum to study controls and subjects with stable COPD and COPD during an infectious exacerbation. In preliminary experiments (Fig. E9 in the Supplementary Appendix), the reducing agent dithioerythritol, which is used to solubilize sputum and thereby make its analysis possible, was shown



not to affect the expression of the invariant natural killer T-cell receptor on human invariant natural killer T-cell clones.

Between 0.2 and 14.3% of the sputum cells were CD3+, of which 27 to 91% were CD4+. As in the bronchoalveolar-lavage analysis, very few invariant natural killer T cells were identified in samples from subjects with mild asthma (0 to 0.1% of all CD3+CD4+ cells) and those with moderately severe asthma (0 to 1.3% of all CD3+CD4+

cells) (Fig. 2 and Table 1). Similarly low counts of invariant natural killer T cells were found in sputum from controls and subjects with COPD (Table 1). Cell counts in sputum from subjects with mild asthma and those with moderately severe asthma were grouped for statistical comparison (with the use of the Mann-Whitney U test) with cell counts in subjects with COPD and controls. No significant differences were found between the groups ($P>0.30$ for the comparison between subjects with asthma

Table 2. Invariant Natural Killer T Cells in Human Airways.*

Sampling Method and Subject No.	Invariant Natural Killer T-Cell Count		
	Vα24+Vβ11+	CD1d Tetramer+	6B11+
	% of CD3+ cells		
Subjects with moderately severe asthma treated with inhaled corticosteroids			
Bronchoalveolar lavage			
2	0.2	0	0
3	0	0.8	0
4	0.1	0.1	0.2
5	0	0	0
6	0	0	0
7	0	0	0.1
8	0	0	0.6
Bronchial biopsy			
4	0	0.4	0.3
7	0	0	0.2
Sputum induction			
15	0	0	0
16	0	0	0.5
Subjects with mild asthma not treated with inhaled corticosteroids			
Bronchoalveolar lavage			
18	0	0.3	2.7
19	0	0	0
Bronchial biopsy			
19	0	0	0
Sputum induction			
19	0	0	0
24	0	0	0
Controls			
Sputum induction			
30	0	0	NA
31	0	0	0
32	0	0	0.1
33	0	0	0.3
34	0	0.1	0.5
35	0	0	0.6
36	0.1	0	0
Subjects with COPD			
Sputum induction			
42	0	0	0
43	0	0.8	0.9
44	0	0	0.8
45	0	0	NA
46	0	0	0

* Percentages of CD3+ T cells that were double-positive for the V α 24 and V β 11 domains or single-positive for CD1d tetramers loaded with α -galactosylceramide or the monoclonal antibody 6B11 are shown. Some subjects underwent sampling by more than one method. NA denotes not analyzed, and COPD chronic obstructive pulmonary disease.

and controls, and $P>0.40$ for the comparison between subjects with stable or exacerbated COPD and controls).

BRONCHIAL-BIOPSY ANALYSIS

Because cells in mucosal tissue could differ from luminal cells, the final flow-cytometric analysis of invariant natural killer T cells was conducted on cells from bronchial-biopsy specimens with the use of enzymatic dispersion with collagenase. We used flow cytometry on dispersed bronchial biopsy-tissue cells to make possible a direct comparison with cells obtained on bronchoalveolar lavage and sputum induction. In addition, flow cytometry permitted the elimination of nonspecifically stained monocytes and macrophages. Because the CD4 epitope on T cells is cleaved during treatment with collagenase,¹⁸ the analysis was restricted to CD3+ T cells. In preliminary experiments (Fig. E9 in the Supplementary Appendix), collagenase treatment was shown not to affect the detection of the $V\alpha 24$ and $V\beta 11$ T-cell receptors on invariant natural killer T-cell clones. Between 0.2 and 7.6% of all cells obtained on biopsy were CD3+ T cells. Consistent with the findings in samples of bronchoalveolar-

lavage cells and sputum, counts of invariant natural killer T cells ranged from 0 to 1.7% of CD3+ T cells (Fig. 3 and Table 1).

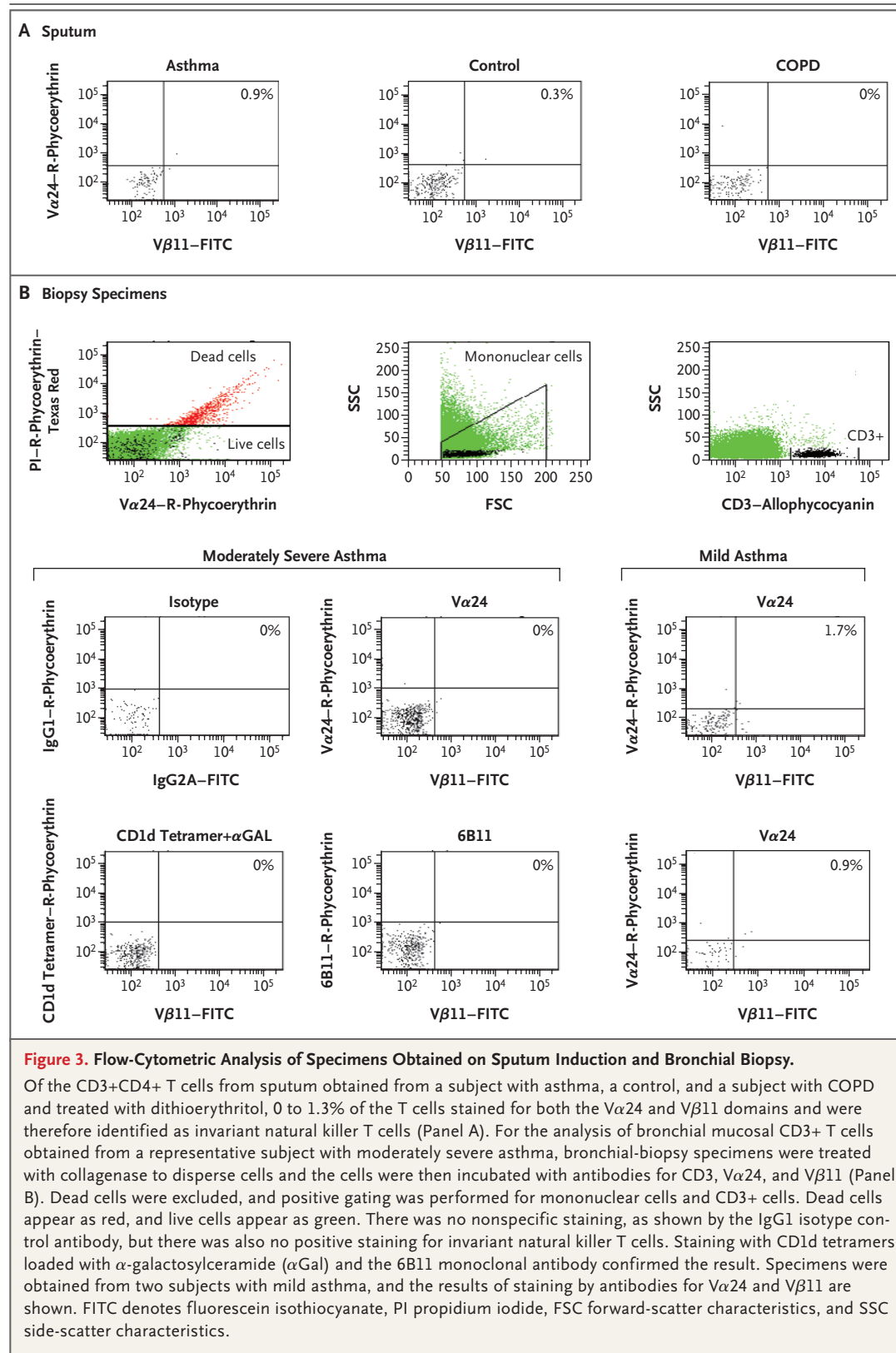
EXPRESSION OF MESSENGER RNA FOR T-CELL RECEPTORS $V\alpha 24$ AND $V\beta 11$

To corroborate the results obtained on flow cytometry with the use of a different method, we performed quantitative (real-time) PCR to detect the messenger RNA (mRNA) for the genes of the T-cell-receptor family, $V\alpha 24$ (*TRAV10*) and $V\beta 11$ (*TRVB25-1*), on bronchoalveolar-lavage cells obtained from eight subjects with asthma and sputum cells obtained from four subjects with asthma, four with COPD, and seven controls. Amplified expression values for mRNA were normalized against signals obtained for the constant chain of the T-cell receptor encoding mRNA. The signal used to normalize the results was lower in the bronchoalveolar-lavage cells than in the invariant natural killer T-cell clones; titration experiments with the use of 10-fold serial dilution of the natural killer T-cell clones showed that the PCR detection kits for the T-cell receptors $V\alpha 24$ and $V\beta 11$ and the constant chain were extremely sensitive,

Table 3. Total and Differential Cell Counts in Bronchoalveolar-Lavage Samples.*

Subject No.	Total Cell Count	Differential Cell Count				
		Macrophages	Neutrophils	Eosinophils	Lymphocytes	Epithelial Cells
	$\times 10^{-6}/ml$			percent		
1	1.29	73.3	1.0	1.8	0.3	23.8
2	22.65	2.8	94.0	<0.1	0.5	2.8
3	1.2	NA	NA	NA	NA	NA
4	1.6	NA	NA	NA	NA	NA
5	3.5	94.5	3.3	0.3	0.5	1.5
6	4.65	83.0	6.3	0.5	0.3	10.0
7	2.0	NA	NA	NA	NA	NA
8	14.4	93.8	1.8	<0.1	<0.1	4.5
17	3.9	79.8	5.8	0.3	4.0	10.3
18	5.0	78.0	14.5	0.8	1.8	5.5
19	0.8	NA	NA	NA	NA	NA
Mean value	5.5	72.17	18.1	0.53	1.1	8.3

* Total cell counts in bronchoalveolar-lavage samples ranged from 0.8×10^6 per milliliter to 22.6×10^6 per milliliter. Differential cell counts were performed manually on 400 cells with the use of cytopsin preparation of bronchoalveolar-lavage fluid and the Rapi-Diff II stain pack (Bios-Europe). Differential cell counts were not analyzed in some subjects, because whole samples were used for extensive validation of flow cytometry and real-time PCR. NA denotes not analyzed.



detecting as few as 10 cells (Fig. E10 in the Supplementary Appendix).

Strong signals for both the constant chain of the T-cell receptor and the mRNA of the individual invariant natural killer T-cell–receptor domains $V\alpha 24$ and $V\beta 11$ were measured in positive control experiments with the use of complementary DNA of invariant natural killer T-cell clones as a template. In contrast, bronchoalveolar-lavage cells from subjects with asthma did not express mRNA for either the $V\alpha 24$ or $V\beta 11$ gene, even though mRNA for the constant chain of the T-cell receptor was readily detected. This finding confirmed the presence of T cells in the samples, but it also ruled out the possibility that invariant natural killer T cells were numerous in bronchoalveolar-lavage fluid from subjects with asthma (Fig. 3). Similar findings were observed with the use of sputum cells (Table E2 in the Supplementary Appendix).

In further experiments, real-time PCR for mRNA expression of the invariant natural killer T-cell receptor ($V\beta 11$) relative to the expression of the constant chain of the T-cell receptor was performed on peripheral blood T cells that had been “spiked” with invariant natural killer T-cell clones so that the samples contained 100%, 60%, and 2% of invariant natural killer T cells. This method generated a “standard curve” for the expression of the $V\beta 11$ gene (*TRVB25-1*) relative to the gene encoding the constant chain of the T-cell receptor. The result established that the proportion of T cells expressing T-cell receptor $V\beta 11$ -encoding mRNA was less than 2% in all samples obtained by bronchoalveolar lavage and sputum induction (Fig. 4).

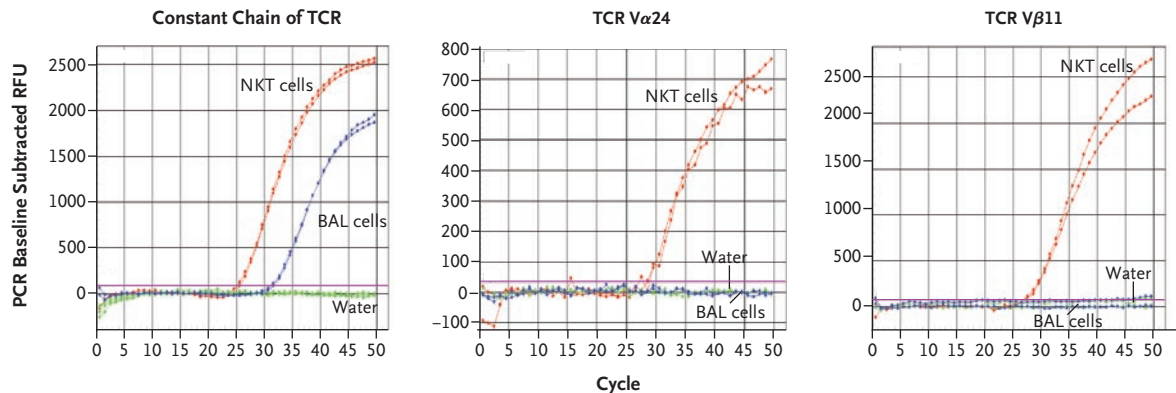
DISCUSSION

Unlike some previous reports, our data show that invariant natural killer T cells are a minority cell population in human lungs of subjects with asthma, constituting less than 2% of the T cells in both the airway lumen (in samples of sputum and bronchoalveolar-lavage fluid) and mucosa (in samples obtained on bronchial biopsy). Moreover, the frequency of invariant natural killer T cells in samples obtained from subjects with asthma did not differ significantly from the frequency in samples obtained from healthy controls or from subjects with stable COPD or COPD during exacerbation.

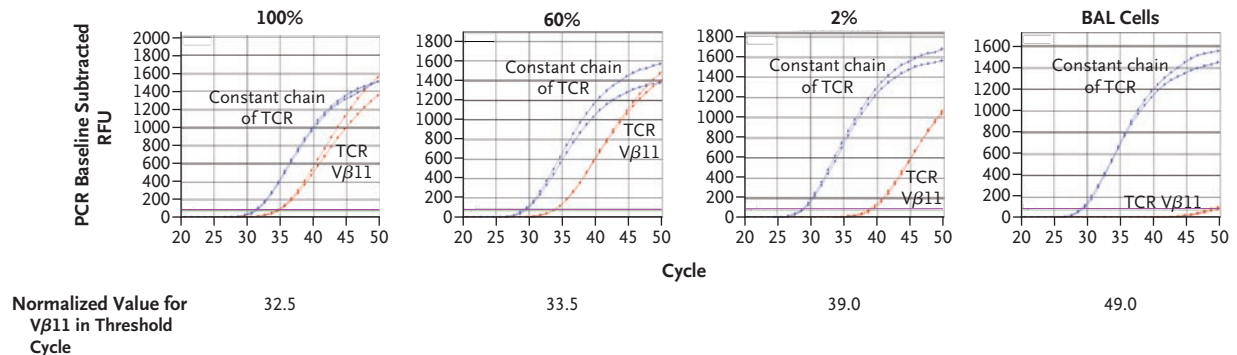
Invariant natural killer T cells are characterized on the basis of their dual staining for the T-cell–

receptor domains $V\alpha 24$ and $V\beta 11$, positive staining with the use of CD1d tetramers loaded with α -galactosylceramide, or both.^{8–10} In this study, we used both criteria in addition to using the 6B11 antibody directed against the invariant $V\alpha 24$ – $J\alpha 18$ region of the invariant natural killer T-cell receptor. Our findings of low counts of invariant natural killer T cells were supported by our inability to detect mRNA for either T-cell–receptor gene $V\alpha 24$ or $V\beta 11$ in samples of bronchoalveolar-lavage fluid or sputum from subjects with asthma (Fig. 4, and Table E2 in the Supplementary Appendix). In addition, we were unable to demonstrate the production of Th2 cytokines by bronchoalveolar-lavage cells from subjects with asthma stimulated with α -galactosylceramide (Supplementary Appendix), the specific stimulus for invariant natural killer T cells. In support of our findings, Thomas et al. found similarly low numbers of invariant natural killer T cells in subjects with asthma not receiving corticosteroids.⁷ Our review of two studies claiming that the numbers of invariant natural killer T cells were elevated in asthma showed that the strict criteria for detecting invariant natural killer T cells were not met in these studies.^{5,19} Although another study⁶ suggested that the numbers of invariant natural killer T cells were slightly elevated in children with severe asthma, the numbers were within the same range as those in our study.

Flow-cytometric analysis of airway T cells is fraught with difficulty because of large numbers of autofluorescent macrophages and cell debris.^{20,21} For this reason, we have excluded dead cells and cell doublets (Fig. 1). Also, we used allophycocyanin as the fluorescent dye to avoid autofluorescence. Alveolar macrophages can nonspecifically bind both antibodies and CD1d tetramers loaded with α -galactosylceramide used to identify invariant natural killer T cells, giving rise to further false positive results on staining. We show that the major fraction of all CD4+ $V\alpha 24$ + cells are identifiable in the nonlymphocyte portion of the forward- and side-scatter plot (Fig. E4 in the Supplementary Appendix) (a finding that can be explained by the presence of CD4 on macrophages) if these cells are not excluded by their typical forward- and side-scatter properties. In contrast, when all measures are used to avoid nonspecific staining and when combining anti-CD3 and anti-CD4 antibodies (Fig. 1, and Fig. E4 in the Supplementary Appendix), CD4+ cells are restricted to the typical lymphocyte cluster in the forward- and side-scatter plot. Simi-

A Real-Time PCR Comparing BAL Cells and iNK T-Cell Clones**B Values in Threshold Cycle in BAL Samples**

Group	Subject No.	Constant Chain of TCR	Variable Chain of TCR	
			Vα24	Vβ11
iNK T-cell clone		27.6	27.2	29.6
Subjects with moderately severe asthma treated with inhaled corticosteroids	1	32.5	—	Not detected
	2	32.3	Not detected	Not detected
	3	32.3	Not detected	Not detected
	5	31.9	Not detected	55.0
	6	31.3	Not detected	Not detected
	7	25.2	Not detected	47.7
Subjects with mild asthma not treated with corticosteroids	17	28.5	Not detected	47.3
	18	33.6	Not detected	Not detected

C Spiking T Cells with iNK T Cells**Figure 4. Real-Time PCR to Detect the Invariant Natural Killer T-Cell Receptor.**

Bronchoalveolar-lavage (BAL) cells were processed into lysis buffer immediately upon recovery and stored at -80°C until real-time PCR analysis with the use of primers for the Vα24 and Vβ11 domains. Real-time PCR for the constant region of the T-cell receptor (TCR) expressed on T cells was used as an internal control for T cells. Red lines represent the invariant natural killer T-cell clones used as a positive control, blue lines represent BAL cells, and green lines represent the negative control (water) (Panel A). BAL cells can be seen not to express mRNA for either domain of the invariant natural killer cell (iNK) TCR but to express the constant chain of the TCR. Values of the threshold cycle of real-time PCR for invariant natural killer T-cell clones and BAL cells from eight subjects with asthma are shown (Panel B). Expression of mRNA for the invariant natural killer TCR was not detected in BAL cells from subjects with asthma. Peripheral-blood T cells were “spiked” with cells from the iNK T-cell clone, and the resultant samples contained 100%, 60%, and 2% of iNK T cells (Panel C). This method generated a “standard curve” for the expression of the Vβ11 gene relative to the gene encoding the constant domain of the TCR. Using this standard curve, we established that the proportion of T cells expressing TCR–Vβ11-encoding mRNA (iNK T cells) was less than 2% in all samples of BAL cells tested (Panel C). The purple line represents the threshold line. RFU denotes relative fluorescence units.

lar problems related to nonspecific binding are encountered with both anti-CD3 antibodies and CD1d tetramers loaded with α -galactosylceramide (Fig. E5 and E6 in the Supplementary Appendix). Thomas et al. have proposed that the use of the 6B11 antibody, which recognizes the invariant complementarity-determining-region 3 loop of the invariant natural killer T-cell receptor, without using anti-V α 24 or anti-V β 11 antibodies is inappropriate.⁷ Therefore, we used a more comprehensive panel of antibodies, including 6B11 monoclonal antibody, anti-V α 24 monoclonal antibody, and anti-V β 11 monoclonal antibody, as well as CD1d tetramers loaded with the invariant natural killer T ligand α -galactosylceramide, and all these consistently showed low counts of invariant natural killer T cells in all analyzed samples.

In summary, in contrast to the report by Akbari et al.,⁴ our study found very few invariant natural killer T cells in the airways of subjects with either mild or moderately severe asthma, subjects with stable COPD or COPD during an exacerbation, and healthy controls. This finding strongly questions

the significance of invariant natural killer T cells in the pathogenesis of asthma. Our data suggest that therapeutic strategies in asthma should therefore continue to be focused on class II MHC-restricted T cells rather than on invariant natural killer T cells.

The study was funded by the University of Southampton.

Mr. Seumois reports receiving grants from the Société Belge de Pneumologie and the Union Chimique Belge Institute for Allergy; Dr. Gadola, consulting fees from Pfizer, lecture fees from Mepha Pharma and Robapharm, and grants from Novartis, Essex, Abbott, and Avidex; Dr. Friedmann, fees for serving on the international safety review panel of Astellas, consulting fees from Schering-Plough, and a grant from GlaxoSmithKline; and Dr. Djukanović, consulting fees from Kyowa Pharmaceuticals, Trinity-Chiesi, and Shionogi, lecture fees from Novartis and AstraZeneca, and grants from GlaxoSmithKline and AstraZeneca. Dr. Powell is the founder of PrimerDesign, which made the primers for the real-time PCR analyses performed in the study, and Dr. Djukanović is one of the three founders of Synairgen. No other potential conflict of interest relevant to this article was reported.

We thank the staff of the Wellcome Trust Clinical Research Facility at Southampton General Hospital, where recruitment, sputum induction, and bronchoscopy were conducted, for their administrative and technical support; Dr. Karl Staples for reviewing the manuscript; and Dr. Laurie Lau for performing the enzyme-linked immunosorbent assay.

REFERENCES

1. Kay AB. Allergy and allergic diseases. *N Engl J Med* 2001;344:30-7.
2. Robinson DS, Hamid Q, Ying S, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298-304.
3. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* 2003; 111:450-63.
4. Akbari O, Faul JL, Hoyte EG, et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N Engl J Med* 2006;354:1117-29.
5. Hamzaoui A, Rouhou SC, Grairi H, et al. NKT cells in the induced sputum of severe asthmatics. *Mediators Inflamm* 2006; 2006:71214.
6. Pham-Thi N, de Blic J, Le Bourgeois M, Dy M, Scheinmann P, Leite-de-Moraes MC. Enhanced frequency of immunoregulatory invariant natural killer T cells in the airways of children with asthma. *J Allergy Clin Immunol* 2006;117:217-8.
7. Thomas SY, Lilly CM, Luster AD. Invariant natural killer T cells in bronchial asthma. *N Engl J Med* 2006;354:2613-5.
8. Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol* 2005;23:877-900.
9. Karadimitris A, Gadola S, Altamirano M, et al. Human CD1d-glycolipid tetramers generated by in vitro oxidative refolding chromatography. *Proc Natl Acad Sci U S A* 2001;98:3294-8.
10. Lucas M, Gadola S, Meier U, et al. Frequency and phenotype of circulating Valpha24/Vbeta11 double-positive natural killer T cells during hepatitis C virus infection. *J Virol* 2003;77:2251-7.
11. Bousquet J. Global Initiative for Asthma (GINA) and its objectives. *Clin Exp Allergy* 2000;30:Suppl 1:2-5.
12. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-76.
13. BTS guidelines for the management of chronic obstructive pulmonary disease. *Thorax* 1997;52:Suppl:S1-S28.
14. Vignola AM, Rennar SI, Hargreave FE, et al. Standardised methodology of sputum induction and processing: future directions. *Eur Respir J Suppl* 2002;37:51s-55s.
15. Djukanović R, Wilson JW, Lai CK, Holgate ST, Howarth PH. The safety aspects of fiberoptic bronchoscopy, bronchoalveolar lavage, and endobronchial biopsy in asthma. *Am Rev Respir Dis* 1991; 143:772-7.
16. Ho LP, Urban BC, Thickett DR, Davies RJ, McMichael AJ. Deficiency of a subset of T-cells with immunoregulatory properties in sarcoidosis. *Lancet* 2005;365:1062-72.
17. Metelitsa LS. Flow cytometry for natural killer T cells: multi-parameter methods for multifunctional cells. *Clin Immunol* 2004;110:267-76.
18. Gunther U, Holloway JA, Gordon JN, et al. Phenotypic characterization of CD3+7+ cells in developing human intestine and an analysis of their ability to differentiate into T cells. *J Immunol* 2005; 174:5414-22. [Erratum, *J Immunol* 2005; 174:8219.]
19. Sen Y, Yongyi B, Yuling H, et al. Valpha 24-invariant NKT cells from patients with allergic asthma express CCR9 at high frequency and induce Th2 bias of CD3+ T cells upon CD226 engagement. *J Immunol* 2005;175:4914-26.
20. Soethout EC, Muller KE, van den Belt AJM, Rutten VPMG. Identification and phenotyping of leukocytes in bovine bronchoalveolar lavage fluid. *Clin Diagn Lab Immunol* 2004;11:795-8.
21. Weyde J, Wassermann K, Schell-Fredrick E. Analysis of single and double-stained alveolar macrophages by flow cytometry. *J Immunol Methods* 1997;207: 115-23.

Copyright © 2007 Massachusetts Medical Society.

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Vijayanand P, Seumois G, Pickard C, et al. Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. *N Engl J Med* 2007;356:1410-22.

TABLE E1. SUBJECT CHARACTERISTICS								
Group	Characteristic						Lung function	
	Patient N°	Age yrs	Sex	Duration of Asthma yrs	Medications Received	Allergic Status	FEV ₁ % of predicted	FEV ₁ : FVC
Moderate asthmatics subjects on inhaled corticosteroids	1	59	F	4	Fluticasone propionate, salmeterol, salbutamol	Parietaria	90	0.58
	2	71	F	33	Fluticasone propionate, salbutamol	Negative	69	0.60
	3	41	M	41	Fluticasone propionate, salmeterol, salbutamol	Dust mite, grass pollen, tree pollen	86	0.66
	4	25	M	20	Beclomethasone diopropionate, salbutamol	Dust mite, grass pollen, cat, dog	85	0.70
	5	42	F	23	Salbutamol, budesonide, formoterol, montelukast	Tree pollen	49	0.55
	6	19	F	18	Salbutamol, Beclomethasone diopropionate, formoterol, montelukast	Dust mite, grass pollen, cat, dog, fungi, cockroach	125	0.95
	7	24	F	8	Fluticasone propionate, salmeterol, salbutamol, cetirizine, montelukast	Dust mite, grass pollen, dog, cat	93	0.82
	8	57	F	6	Fluticasone propionate, salmeterol, salbutamol, montelukast, ceterizine	Negative	119	0.89
	9	49	F	44	Fluticasone propionate, salmeterol	Dust mite, grass pollen, cat	94	0.68
	10	47	M	11	Beclomethasone diopropionate, salbutamol	Dust mite, tree pollen, cat	118	0.75
	11	21	F	17	Budesonide, terbutaline sulphate	Dust mite, grass pollen	107	0.84

	12	36	F	24	Beclomethasone diopropionate, salbutamol	Dust mite	73	0.59
	13	59	M	9	Budesonide, formoterol fumarate	Dust mite, grass pollen, tree pollen	93	0.72
	14	55	M	55	Beclomethasone diopropionate, salbutamol	Dust mite	98	0.57
	15	47	M	42	Budesonide, formoterol	Grass pollen	79	0.81
	16	49	M	44	Budesonide, formoterol	Grass pollen	68	0.68
Patients with asthma not treated with corticosteroids	17	55	F	5	Salbutamol	Dust mite, dog	94	0.69
	18	37	F	15	Salbutamol	Dust mite, cat, dog	101	0.81
	19	30	F	18	Salbutamol	Dust mite, grass pollen, tree pollen, dog, cat	89	0.82
	20	22	M	22	Salbutamol	Dust mite, dog	94	0.81
	21	58	M	58	Salbutamol	Dust mite, dog	84	0.73
	22	19	F	14	Salbutamol	Dust mite, grass pollen, tree pollen, cat	128	0.85
	23	20	M	10	Salbutamol	Dust mite, tree pollen	87	0.63
	24	24	M	14	Salbutamol	Dust mite	101	0.85

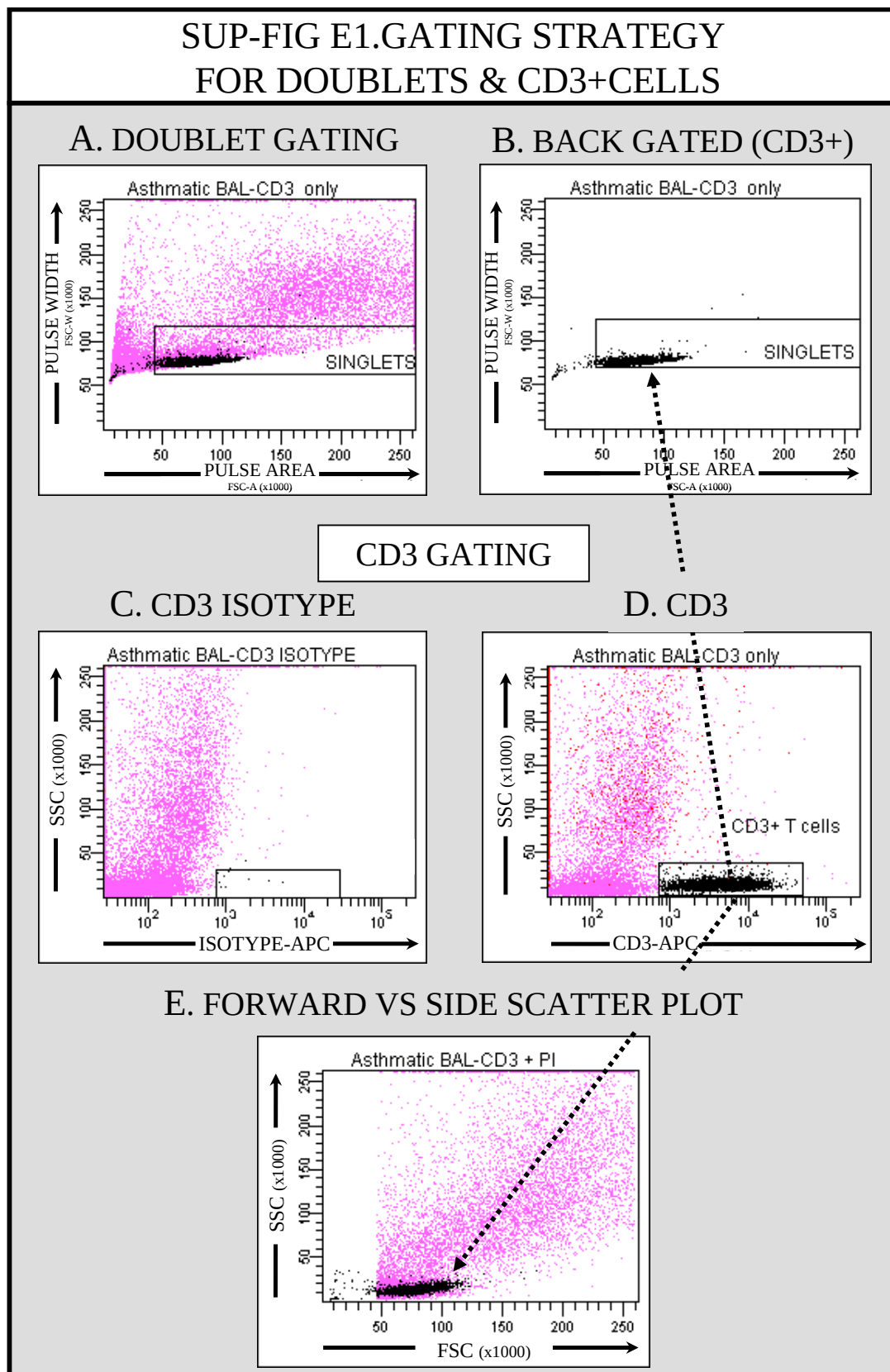
Healthy controls	25	21	M	-	None	Grass pollen, dust mite	107	0.87
	26	33	M	-	None	Negative	110	0.82
	27	36	M	-	None	Grass pollen	104	0.70
	28	40	F	-	None	Grass pollen	90	0.74
	29	51	M	-	None	Negative	103	0.78
	30	19	M	-	None	Dust mite	121	0.81
	31	49	M	-	None	Grass pollen	120	0.86
	32	56	M	-	None	Negative	102	0.76
	33	59	M	-	None	Negative	99	0.68
	34	39	M	-	None	Grass pollen, cat	111	0.83
	35	64	F	-	Levothyroxine, statin, aspirin	Negative	92	0.71
	36	28	M	-	None	Cat	100	0.89

Patients with COPD exacerbation	37	79	M	-	Salbutamol, ipratropium bromide, fluticasone propionate, salmeterol	-	9	0.45
	38	79	F	-	Fluticasone propionate, salmeterol, salbutamol, ipratropium bromide	-	23	0.86
	39	70	F	-	Fluticasone propionate, salmeterol, salbutamol, ipratropium bromide	-	17	0.65
	40	71	M	-	Fluticasone propionate, salmeterol, salbutamol, tiotropium bromide, theophylline	-	18	0.77
	41	48	F	-	Beclomethasone diopropionate salbutamol, ipratropium bromide	-	15	0.68
Patients with stable COPD	42	71	M	-	Salbutamol, ipratropium bromide, fluticasone propionate, salmeterol	-	41	0.53
	44	65	M	-	Aspirin, simvastatin, bendrofluazide, lisinopril	-	92	0.65
	44	79	F	-	Salbutamol, ipratropium bromide, fluticasone propionate, salmeterol prednisolone, carbamazepine	-	29	0.69
	45	50	F	-	Beclomethasone diopropionate, pantoprazole, salmeterol, salbutamol	-	130	0.73
	46	72	M	-	Fluticasone propionate, salmeterol, salbutamol, ipratropium bromide	-	48	0.57

Forty six subjects in total participated in this study, including 16 moderate asthmatics, 8 mild asthmatics, 10 patients with COPD and 12 healthy control subjects. Their details, including age, gender and lung function for all subjects, duration of symptoms for the asthmatics, and medication for the asthmatic and COPD patients are shown in table E-1.

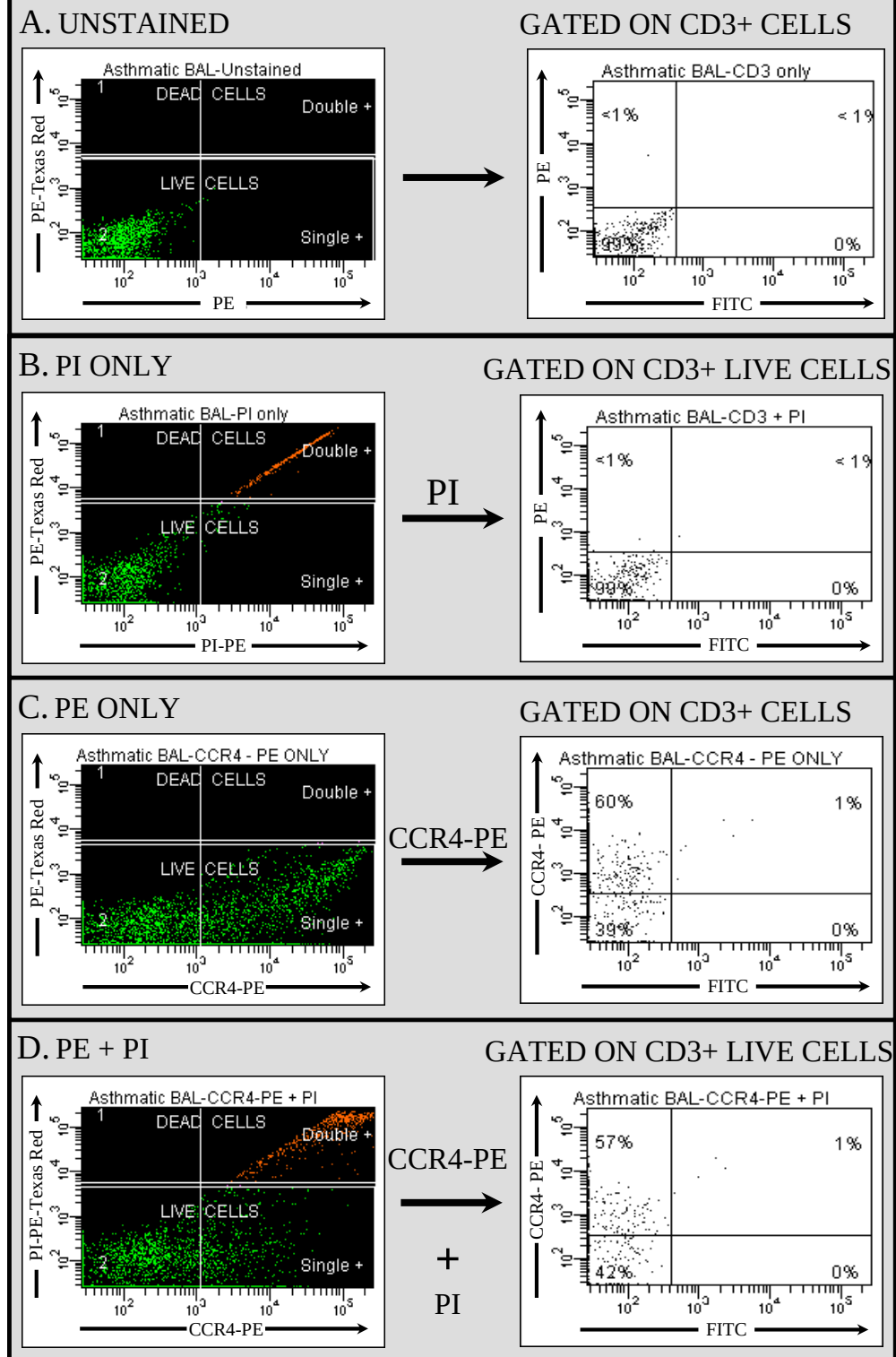
Justification of the gating strategy for detecting iNKT cells in airway samples. This text is accompanied by Supplemental Figure E1 to which it refers.

Initially, cell doublets, which can contain adherent cells (e.g. macrophages) or dead cells, and thus bind antibodies non-specifically, are gated out using pulse width and area (SUP-figure E1A) (also see figure 1B in main text) by excluding cells with area less than 50×10^3 and pulse width greater than 120×10^3 . Dead cells are further excluded by virtue of their staining with the DNA-binding agent, propidium iodide (also see figure 1C and SUP-figure E2). Large cells and debris are then excluded in the forward and side-scatter plot (figure 1D in the main text), although some large cells (e.g. epithelial cells and macrophages) are already excluded by doublet gating. CD3⁺ T cells are gated positively by their staining with anti-CD3 antibody conjugated to allophycocyanin (APC), a fluorescent dye which emits light of a wavelength that is beyond the range of light emitted by autofluorescent cells (macrophages). As expected, after backgating these CD3⁺ cells are found as a tight group of cells within the singlet gate (see black-coloured dots (cells) in SUP-figure E1B) and within the lymphocyte cluster of the forward and side scatter plot (SSC) (SUP-figure E1E). Thus, we show that neither the exclusion of doublets nor the selection of CD3⁺ cells with low SSC excludes lymphocytes which contain the iNKT population.



SUP-FigureE1. Gating strategy for cell doublet exclusion and CD3+ cell identification.

SUP-FIG E2. GATING STRATEGY FOR EXCLUDING DEAD CELLS

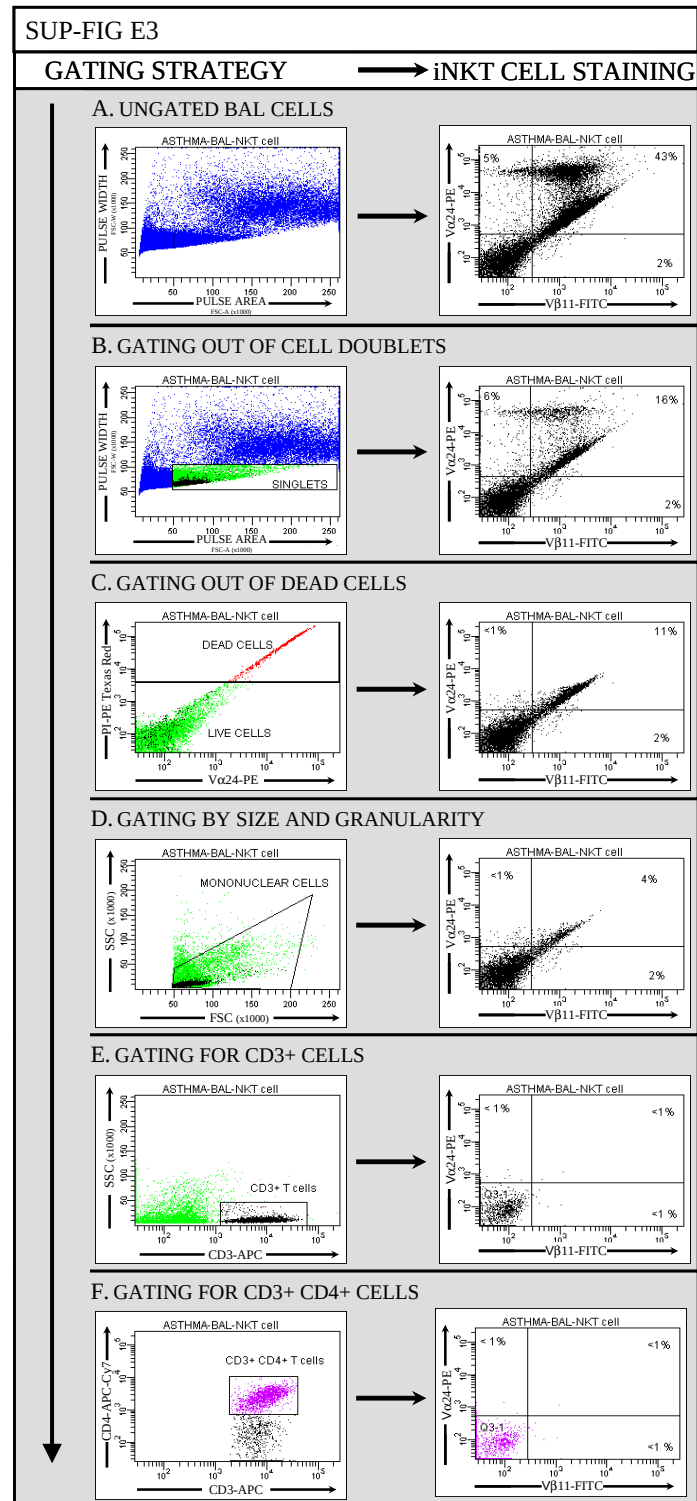


SUP-Figure E2. Gating strategy for excluding dead cells.

Flow cytometric analysis is done using the 7-colour FACSAria™ which has the facility to discriminate between R-Phycoerythrin (PE)- and propidium iodide (PI)-stained cells by virtue of its 7 detectors. In particular, the PE-Texas Red detector allows separation of PI positive dead cells from PE positive cells by taking advantage of the spectral overlap of PI in both the PE and PE-Texas Red detectors. Thus, PI positive dead cells are double positive cells when looking in the PE and PE-Texas Red channels (E2B), whereas cells stained with PE-conjugated antibody are single positive (E2C). We show the sequential gating for dead cells (panels on the left side) and live CD3⁺ cells (panels on the right side; please note that the FITC-conjugated antibody is not used here). We start by displaying the unstained cells (see first row E2A) and set the first gate. Using PE and PE-Texas Red, we identify the population of dead cells which are PE⁺/PE-Texas Red⁺. Only these double positive cells are removed from further analysis while retaining the PE⁺/PE-Texas Red⁻ live fraction. When using PE-conjugated anti-CCR4 antibody (C-C chemokine receptor 4 (CCR4) is expressed predominantly in Th2 T cells in asthmatic lungs¹), we can see that these cells constitute 60% of CD3⁺ T cells in asthmatic BAL (E2C right panel). We also show that the removal of PE⁺/PE-Texas Red⁺ dead cells has virtually no effect on the frequency of CCR4⁺ cells (E2D). The slight reduction of the CCR4⁺ count by only 3% is due to non-specific binding of anti-CCR4 antibody to dead cells. Overall, this suggests that the gating strategy for excluding dead cells using PI does not affect the sensitivity of T cell subtype detection (in the PE channel) but improves the specificity.

SUP-FigureE3 shows the gating strategy when analysing staining for Vα24 and Vβ11antibodies. It can be seen that, as is the case with the analysis using CD1d

Tetramer+ α Gal (figure 1 in the article), when stringent gating is not applied, erroneously high percentages of iNKT cells can be detected.



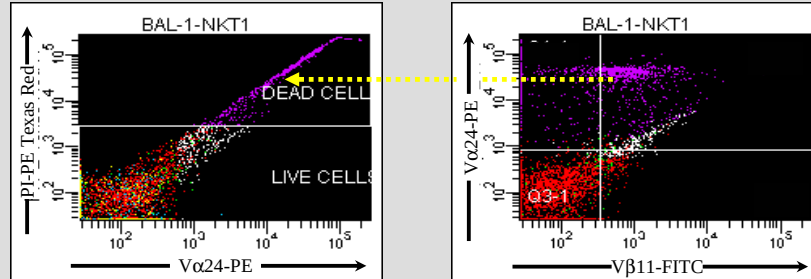
SUP-Figure E3: BAL cell analysis for the surface expression of the invariant NKT T cell receptor (TCR). BAL cells were separated from the fluid phase and incubated in the presence of antibodies for CD3 (APC-conjugated), CD4 (APC-Cy7-conjugated) and the invariant TCR of iNKT cells (V α 24 and V β 11) (PE- and FITC-conjugated, respectively) and analysed by 7-colour flow cytometer (FACSAriaTM). The left column of figures indicates the gating strategy. After viewing all ungated cells (E3A), cell doublets (E3B) and dead cells (staining with propidium iodide PI) (E3C) were gated out of further analysis in order to remove non-specifically stained cells. Mononuclear cells (E3D) were gated by size and granularity on forward and side scatter, respectively. Helper/inducer CD4⁺ T cells were then gated using a combination of anti-CD3 (E3E) and anti-CD4 (E3F) antibodies. Such serial gating reduced the numbers of cells that could be mistaken for iNKT cells on account of their apparent positivity for the two invariant TCR domains (see the right column of figures under the heading NKT cell staining showing a reduction in apparent NKT cell counts from 43% to <1%), leaving only a small percentage of cells truly positive by either antibody (E3F).

False positive staining for iNKT cells and control for autofluorescence and non-specific binding of antibodies

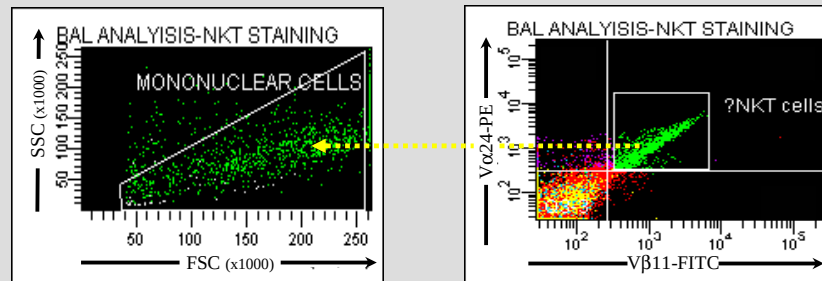
Analysis of BAL cells by flow cytometry is made difficult by the presence of auto-fluorescent alveolar macrophages which are present in large numbers in the recovered BAL fluid. Autofluorescent macrophages can also bind non-specifically both the antibodies and the CD1d Tetramer+ α Gal used for the identification of iNKT cells, giving further rise to false positive staining. Autofluorescence can be minimised by using a fluorescent dye, such as APC, which emits light of a different wave-length to that emitted by auto-fluorescent cells. The non-specific binding of antibodies to Fc γ receptors is best dealt with by using high concentrations of polyclonal human serum IgG or high concentration serum. In this study, we have used 2 mg/ml polyclonal human IgG (Sigma, Poole, UK). Finally, the use of side and forward scatter properties (measures of granularity and cell size, respectively) allows gating out of macrophages which can also stain specifically for CD4 (because they express surface CD4) and non-specifically for CD3 by binding to Fc γ receptor. In SUP-figure E4C we show that, unless gating is done on scatter properties, high numbers of CD4⁺ cells that stain for V α 24 can be back-gated into the non-lymphocyte part of the forward and side-scatter plot. In contrast, when applying the measures to rule out autofluorescence and non-specific binding of antibodies, and using the combination of anti-CD3 and anti-CD4 antibodies, as shown in figure 1E, F of the main text and SUP-figure E4C, CD4⁺ cells are restricted to the typical lymphocyte cluster that is seen in the forward and side-scatter plot. Similar problems with non-specific binding and false detection of iNKT cells are encountered with both anti-CD3 antibodies and CD1d tetramers loaded with α -galactosylceramide unless stringent gating is followed as shown in SUP-figures E5 and E6.

SUP-FIG E4. FALSE POSITIVE DETECTION OF iNKT CELLS

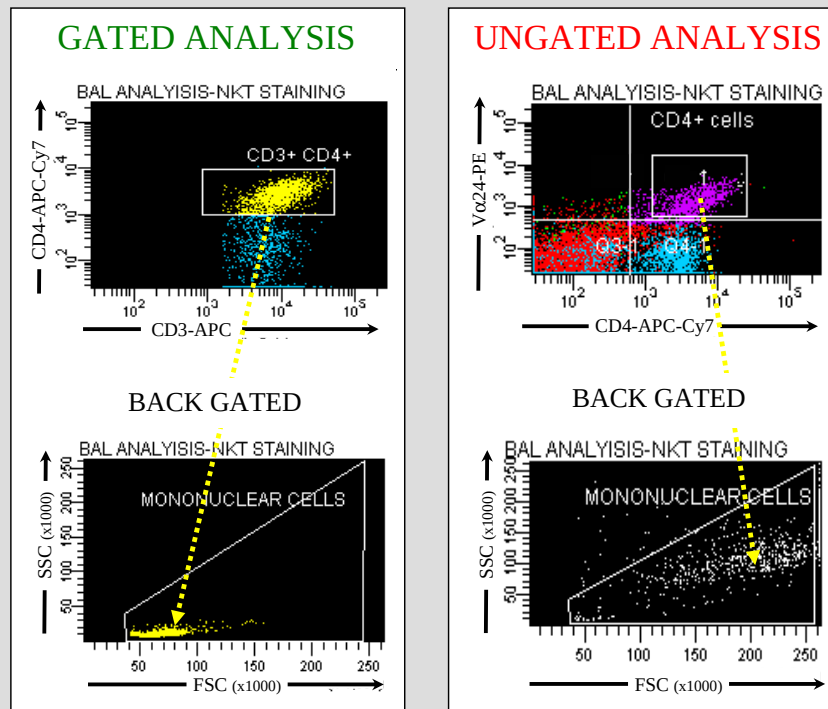
A. DEAD CELLS STAIN WITH iNKT ANTIBODIES



B. MACROPHAGE AUTOFLUORESCENCE



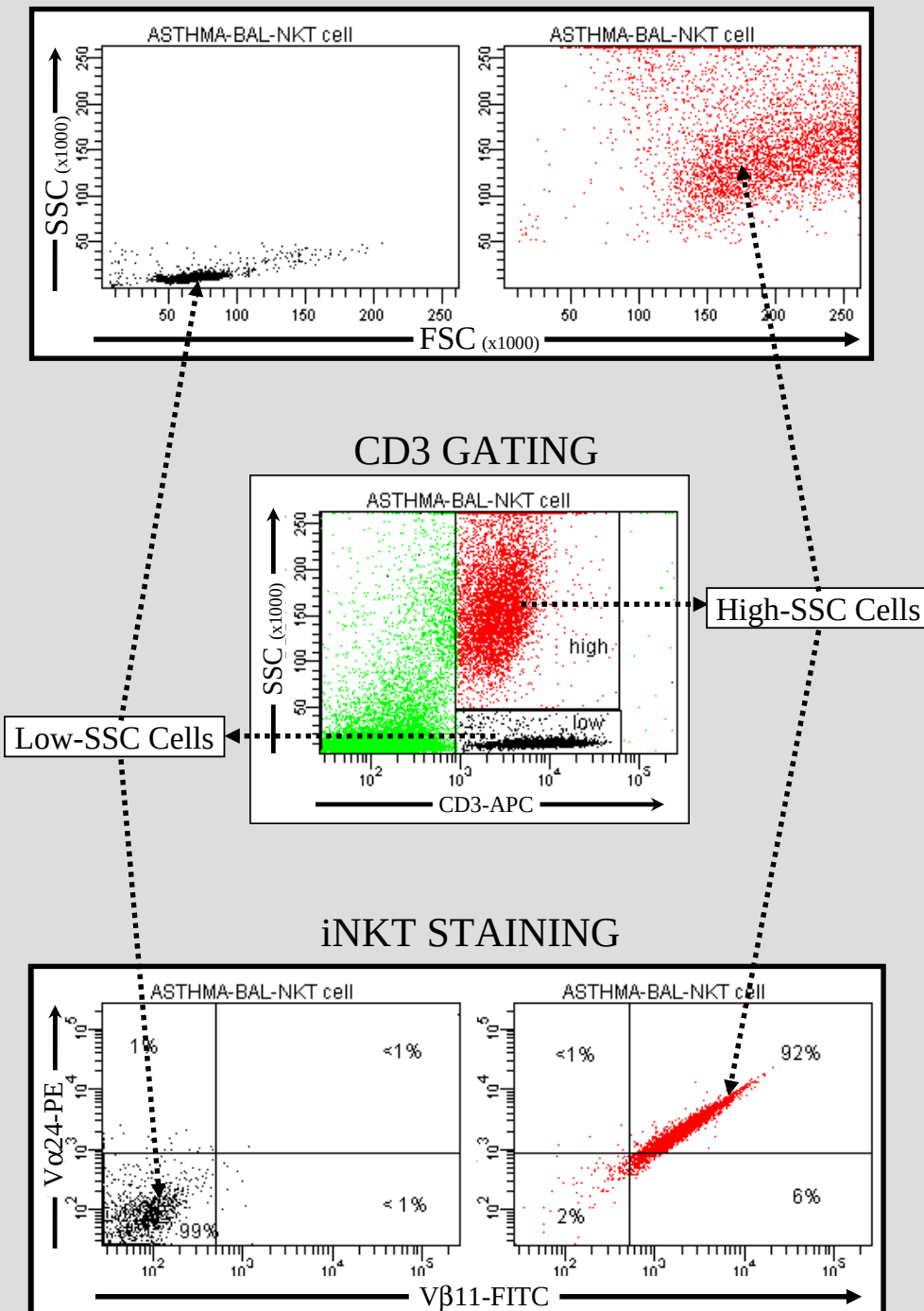
C. FALSE POSITIVE NKT CELLS IN CD4+ CELLS



SUP- Figure E4. False positive staining for iNKT cells due to non-specific binding of antibodies by dead cells (A), and autofluorescent macrophages (B). After initial gating out of cell doublets and dead cells, gating of lymphocytes by low side-scatter (see figure 1E in the main text of the article) is required. If this is not done, cells that autofluoresce and bind antibodies non-specifically can be back-gated and, thereby, shown to have forward and side-scatter properties of macrophages (B). It is critical that following analysis of specific staining, the cell population that is believed to be iNKT cells can be back-gated into the lymphocyte cluster on the forward and side-scatter (as shown in C left panels) and not into a broad cluster which also includes large cells, i.e. macrophages (as shown in C, right panels).

SUP-FIG E5. HIGH-SSC & CD3+ FALSE POSITIVE NKT STAINING

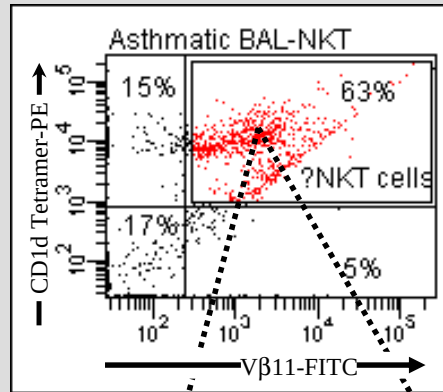
BACK GATING IN FORWARD vs SIDE SCATTER PLOT



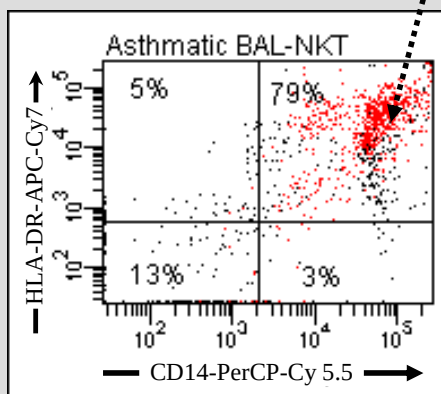
SUP-Figure E5. False positive iNKT detection seen with high side-scatter (SSC) CD3⁺ cells. CD3 gating of BAL cells from an asthmatic subject based on SSC shows two distinct populations: one with a low SSC (shown as black dots) and the other with a high SSC (shown as red dots – see middle plot). Back-gating the low SSC CD3⁺ population (black dots) in the forward and side-scatter plot shows that they fall in the characteristic lymphocyte cluster (top left). On the other hand, the high SSC CD3⁺ population (red dots) falls in the region of macrophages, as shown by their large size in forward scatter (top right). Additionally the high SSC CD3⁺ cells (red dots) are intensely autofluorescent in FITC and PE channels (bottom panel right plot) and/or exhibit non-specific binding to the NKT detection antibodies. In contrast, the low SSC CD3⁺ cells show no autofluorescence and <1% bind to the iNKT detection antibodies (bottom left).

SUP-FIG E6. FALSE POSITIVE iNKT STAINING WITHOUT ANY GATING

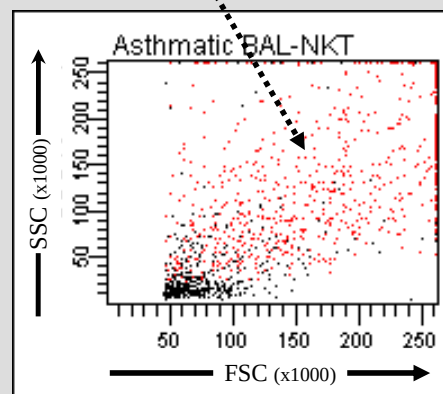
A. CD1d Tetramer + α Gal & V β 11



B. CD14 & HLA-DR



C. FORWARD vs SIDE SCATTER PLOT

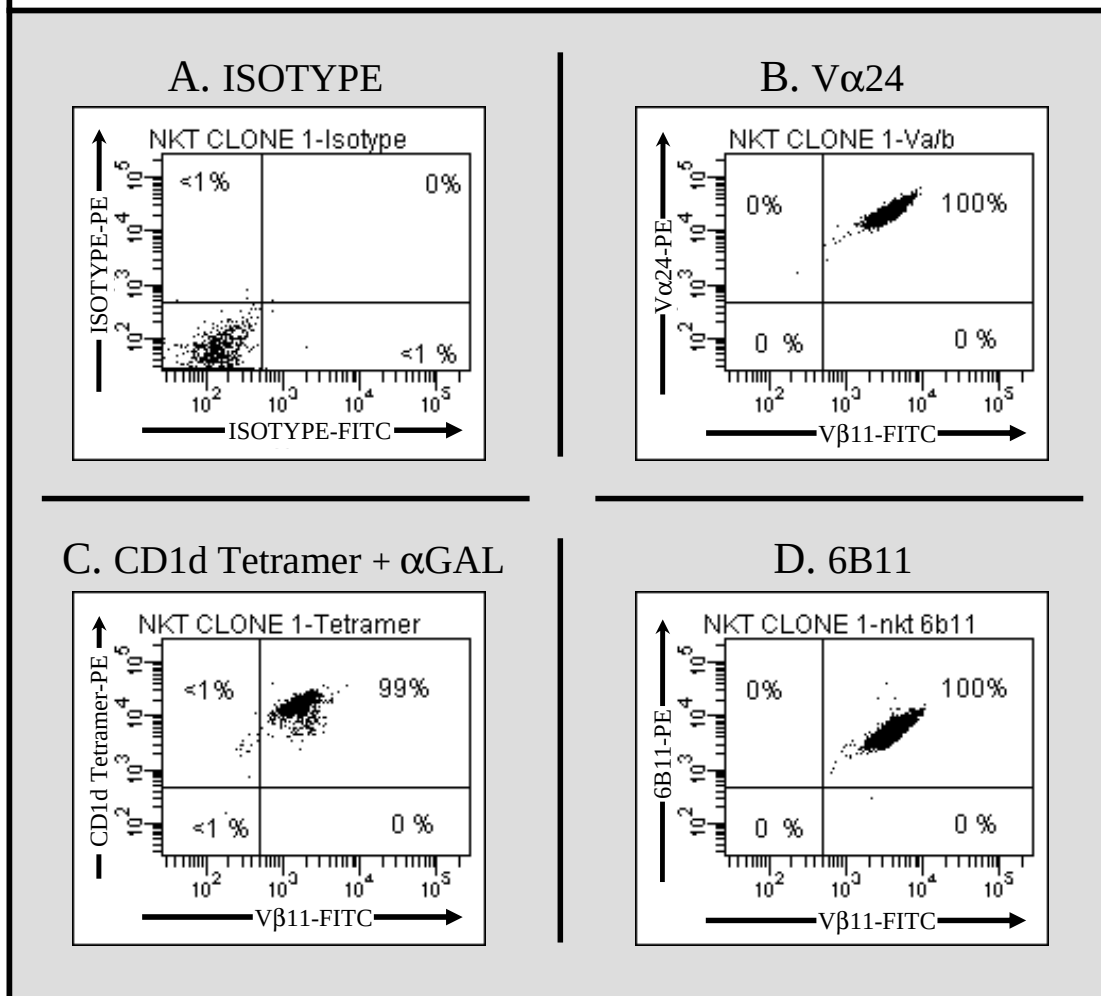


SUP-Figure E6. False positive iNKT cell detection by CD1d Tetramer+ α Gal. When gating procedures, as described in figure 1, are not applied, a significant fraction of cells in BAL samples are stained with CD1d Tetramer+ α Gal and also other antibodies (antiV β 11) (A). However, back-gating of these CD1d Tetramer+ α Gal cells reveals that they are not lymphocytes but are monocyte/macrophages, as shown by intense staining for CD14 and HLA-DR (red dots in B). Also, these cells display a high forward and side-scatter, again suggestive that they are macrophages (red dots in C).

Development and validation of the iNKT cell line

Peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy individuals and the cells stained with antibodies to detect V α 24 and V β 11 T cell receptor on iNKT cells. The double positive cells (iNKT cells) were sorted using FACSARIA™ (BD Biosciences, Oxford, U.K) at 1 cell/well into a 96-well plate containing 2x10⁴ autologous irradiated (2500 RAD) PBMC. Viable iNKT cells were expanded in the presence of phytohaemagglutinin (PHA-p Sigma, Poole, U.K) at 1 μ g/ml and 100 IU/ml of recombinant interleukin-2 (IL-2) (Peprotech, London, U.K). The phenotype of the iNKT cells clone was confirmed by flow cytometric analysis using anti-V α 24, anti-V β 11, anti-6B11 antibodies and CD1d Tetramer+ α Gal (SUP figure E7).

SUP-FIG E7. VALIDATION OF iNKT CELL CLONE

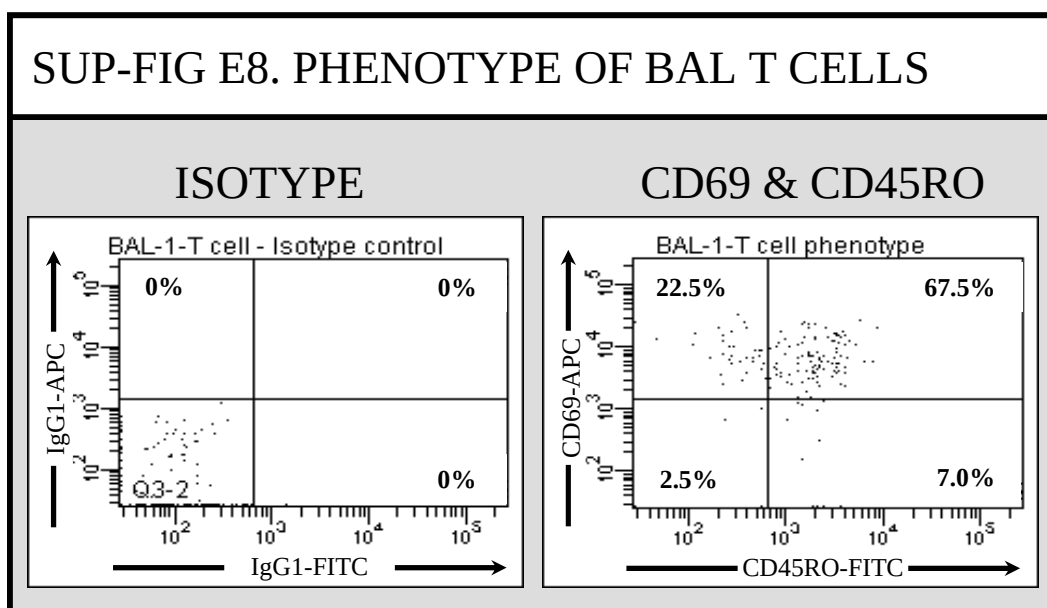


SUP-Figure E7. Validation of the iNKT cell clone and antibodies used in its detection

Cells from the human iNKT cell clone uniformly showed positive staining with antibodies for Vα24 and Vβ11 domains of the TCR (B). Similar uniform staining is also shown with 6B11 antibodies (D) and CD1d Tetramer+αGal (C). Isotype controls are shown in A.

Flow cytometric analysis of BAL cells for activation/memory markers

Flow cytometric analysis of BAL cells in a patient with asthma showed that 74.5% of T cells are CD45RO+, indicating their memory phenotype and that 90% of them are activated, expressing surface CD69. A typical FACS plot of T cells from an asthmatic subject is shown in SUP-Figure E8.



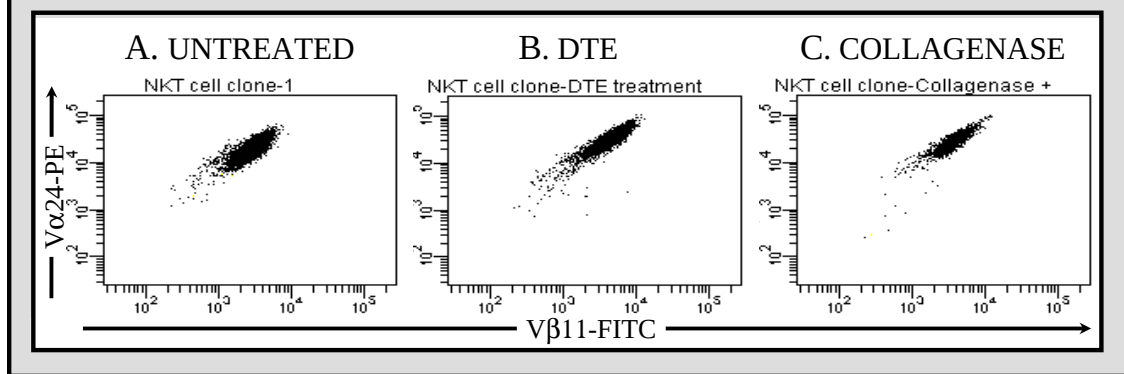
SUP-Figure E8. Flow cytometric analysis of BAL cells showing that 74.5% of T cells are CD45RO+, indicating their memory phenotype, and that 90% of them are activated, expressing surface CD69. (Abbreviations in the figure IgG1: Immunoglobulin G1)

Validation of flow cytometry for the identification of iNKT cells in induced sputum and bronchial biopsies

In preliminary experiments the effects of treatment of sputum with dithioerythritol (DTE) (Sigma, Poole, UK) to ensure that this mucolytic (reducing) agent, used to solubilise sputum and, thereby, enable its analysis, did not have an effect on the expression of the iNKT invariant TCR. The human iNKT cell clone was incubated for 30 min in 0.63 M DTE (routinely used to solubilise sputum) and then stained with antibodies to detect V α 24 and V β 11 T cell receptor on iNKT cells. As shown in SUP-figure E9A,B, DTE had no effect on the expression of either of these two invariant TCR chains.

A similar validation process was required for the analysis of iNKT cells in the bronchial mucosa treated by enzymatic dispersion with collagenase. Bronchial biopsies obtained by fiberoptic bronchoscopy were placed into RPMI medium to which collagenase I (Sigma, Poole, UK) was added at 1 mg/ml. Digestion was allowed to continue for 60 min at 37°C with gentle agitation following which the cells were washed twice in PBS and filtered through a 70- μ m filter. Cells were then incubated for 30 min at 4°C with purified polyclonal human serum IgG to reduce non-specific staining by blocking the Fc γ receptor. Specific antibodies and isotype controls were added as described in the methods section. Given that the CD4 epitope on T cells is cleaved during collagenase treatment², analysis was restricted to CD3⁺ T cells. As shown in figure E9C, collagenase treatment of the iNKT cell clone was shown not to affect the detection of either V α 24 or V β 11 T cell receptor on iNKT cells.

SUP-FIG E9



SUP-Figure E9. Flow cytometric analysis of the iNKT clone cells. Cells from the human iNKT cell clone (A) uniformly showed positive staining with antibodies for Vα24 and Vβ11 domains of the TCR. Surface expression of Vα24 and Vβ11 domains of the TCR on the iNKT cell clone was unaffected by prior treatment with DTE (B) and Collagenase (C).

Expression of iNKT cell receptor mRNA

Real Time PCR was performed using detection kits for the following target genes:

1. TCR V α 24 (TRAV10 gene):

F- AAGCATCTGACGACCTTCTTG, R-AACAGGACCTCTCCCAGTATC

2. TCR V β 11 (TRBV25-1 gene):

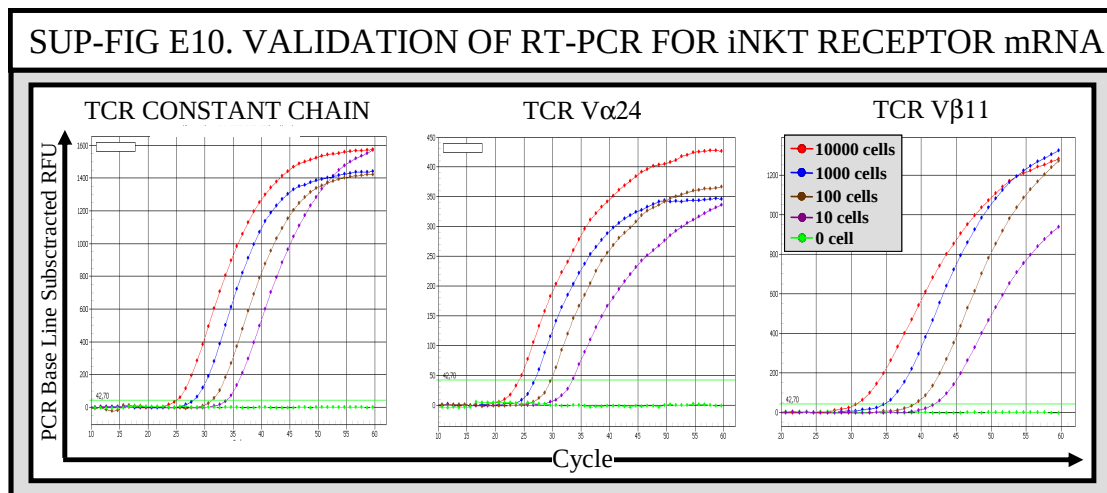
F- GCACAGTTTGGGCTTTTATTTT, R-GAGAACATTCCAGAGTGATCTTC

Both primer set were obtained from Primer Design Ltd (Southampton, UK).

Detection kits for the TCR constant chain (TRBC2 gene) F- CAGCGAGCCCTACTCAAATTAG R-GACCTGTGGAAGAGAGAACATT was also obtained so as to generate a normalising signal for TCR expression. Primers were aligned by BLAST search on the NCBI database with other variable regions to confirm that there was no significant cross-homology with any other variable regions (data not shown).

In the iNKT cell clone, used as a positive control, strong signals were obtained for the TCR constant chain messenger RNA (mRNA) and invariant NKT TCR mRNA (V α 24 and V β 11). In contrast, the asthmatic BAL cells did not express mRNA for the invariant NKT cell receptor (V α 24 and V β 11) even though mRNA for the TCR constant chain was detected, confirming the presence of T cells in these samples but the absence of iNKT cells. It was also noted that the normalising control signal from the TCR constant chain was relatively lower in the BAL cells as compared with the iNKT cell clone. However, a standard curve using 10-fold serial dilutions of iNKT cells established that the PCR

detection kits for TCR V α 24, V β 11 and constant chain could detect as few as 10 cells (SUP-figure E10).



SUP-Figure E10. Validation of Real Time RT-PCR for the V α 24 and V β 11 domains of the invariant TCR expressed on iNKT cells and the constant chain of the TCR expressed on all T cells. Serial cell concentrations (from 10,000 down to 10 cells as shown in red, blue, brown and purple) of the iNKT cell clone were prepared, placed into lysis buffer and subjected to Real Time PCR analysis using the icyclerIQ platform (Bio-Rad, Hemel Hempstead, UK) using primers described in the methods section and the online supplement. The green line represents water.

Similarly, RT-PCR analysis was performed on sputum cells obtained from asthmatic subjects, healthy controls and COPD subjects. Again low signals were detected for the invariant NKT cell receptor (V α 24 and V β 11) even though mRNA for the TCR constant chain was detected, confirming the presence of T cells in these samples but indicating very low proportions of iNKT cells. The threshold cycle values (Ct) for detection of TCR constant chain messenger RNA (mRNA) and invariant NKT TCR mRNA (V α 24 and V β 11) are shown in table E2.

TABLE E2. TRESHOLD CYCLE (Ct) VALUES IN SPUTUM SAMPLES				
Group	Subject No	TCR Constant Chain	TCR Variable Chain	
			V α 24	V β 11
iNKT cell clone		27.6	27.2	29.6
Moderate asthmatic subjects on inhaled corticosteroids	7	28.8	n.d.	n.d.
	15	35.3	n.d.	n.d.
	16	30.1	n.d.	37.1
Mild asthmatic subjects, steroid-naïve	24	40.5	n.d.	n.d.
Healthy subjects	30	35.7	n.d.	n.d.
	31	32.1	n.d.	45.5
	32	31.9	49.7	48.3
	33	31.2	n.d.	38.3
	34	29.9	n.d.	48.5
	35	30.4	39.8	46.4
	36	30.7	36.9	42.1
COPD subjects	43	33.8	n.d.	42.4
	44	33.8	n.d.	43.6
	45	33.5	n.d.	n.d.
	46	31.2	n.d.	n.d.

SUP-Table E2: RT-PCR analysis on sputum cells.

RT-PCR analysis for the detection of iNKT cells in sputum cells of mild and moderately severe asthmatics, subjects with COPD and healthy control subjects. The threshold cycle (Ct) value for the detection of TCR constant chain messenger RNA (mRNA) and invariant NKT TCR mRNA (V α 24 and V β 11) is shown. Low signals for iNKT cell specific mRNA were detected in the sputum samples (either not detected at all [n.d.], or with Ct values at least 6 cycles higher than the Ct for the constant TCR gene expression, which corresponds to <2% of CD3+ cells). n.d. = not detected.

Stimulation of BAL cells with α -galactosylceramide

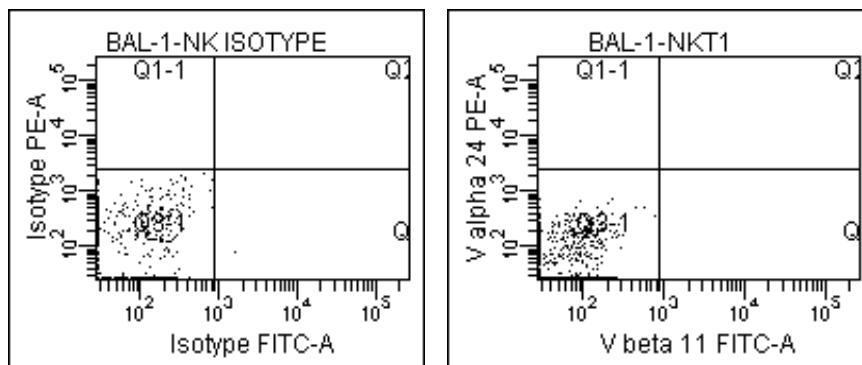
We have attempted to reproduce the *in vitro* findings of Akbari et al.³ by stimulating BAL cells, obtained by bronchoscopy from 4 asthmatic subjects, with α -galactosylceramide for a period of 24 hours. Supernatants were assessed for IL-5 and IL-13. Given that no details of time-course or dose-responses were provided by Akbari and colleagues in their paper, we applied a protocol which we felt should be able to show adequate cytokine responses by iNKT cells without requiring longer-term culture, i.e. typically 5 to 7 days for cultures of PBMC stimulated with allergen.

BAL cells (2×10^6 /ml) were cultured in cell growth medium (RPMI 1640 supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin G and streptomycin) using 96-well U bottom plates. The following conditions were set up: 1) medium plus vehicle for α -galactosylceramide only, 2) α -galactosylceramide (100 ng/ml) (standard dose used by others⁴, 3) α -galactosylceramide (500 ng/ml) (higher dose), 4) α -galactosylceramide (100 ng/ml) + anti-human CD1d blocking antibody (25 μ g/ml, clone CD1d42, mouse IgG1 kappa (BD PharmingenTM) to ensure CD1d restriction of measured cytokine responses, 5) α -galactosylceramide (100 ng/ml) + mouse IgG1 kappa isotype control antibody (25 μ g/ml (BD Pharmingen), 6) β -galactosylceramide (100 ng/ml), as a negative control lipid, 7) house dust mite allergen at 5000 SQ-U/ml (*Dermatophagoides pteronyssinus*, ALK-Abello A/S, Horsholm, Denmark) for comparison with a standard Th2 stimulus in asthmatic patients, and, 8) anti-CD3/CD28 (1 μ g/ml) as a pan-T-cell stimulus. BAL samples from two patients with mild asthma and 1 with moderately severe asthma were cultured using all the above conditions and samples from 1 moderately severe asthmatic were cultured under conditions 1, 2, 3, 6, 7, and 8. No release of either IL-5 or IL-13 was detected following 24-hour stimulation of BAL cells from asthmatic subjects

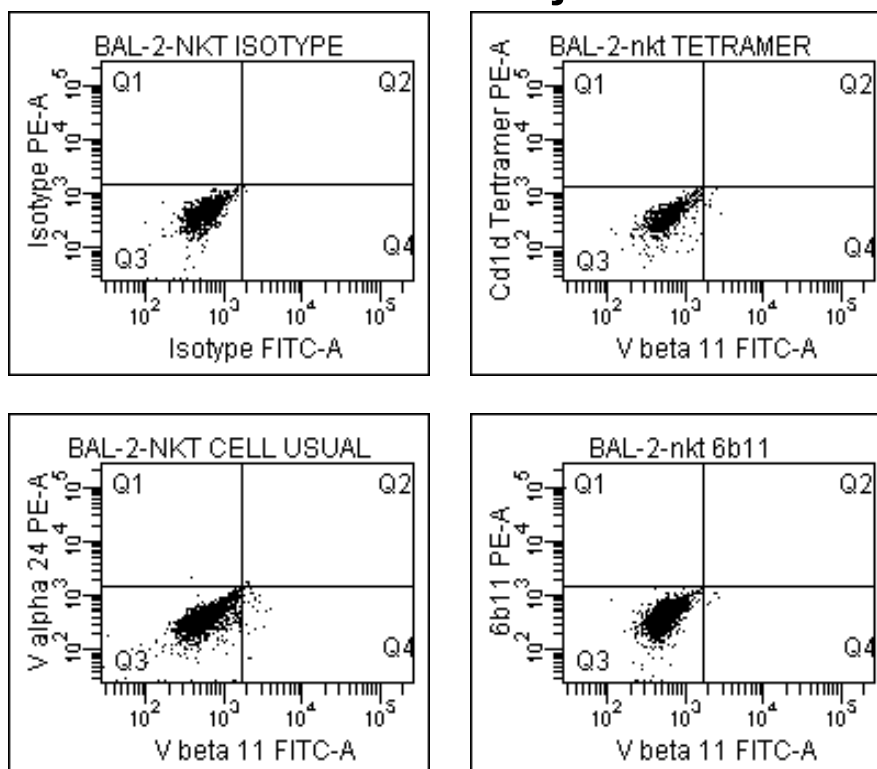
with 1) α -galactosylceramide, 2) the irrelevant lipid, β -galactosylceramide, 3) house dust mite allergen extract or 4) anti-CD3/CD28 antibody. None of these subjects had detectable iNKT cells in their BAL by flow cytometry.

Flow cytometric analyses using 6B11 and CD1d Tetramer+ α Gal were performed on BAL samples from 9 asthmatic subjects. These are shown below as individual plots for all the subjects together with the results of double staining for V α 24 and V β 11 and isotype control (SUP-figure E11).

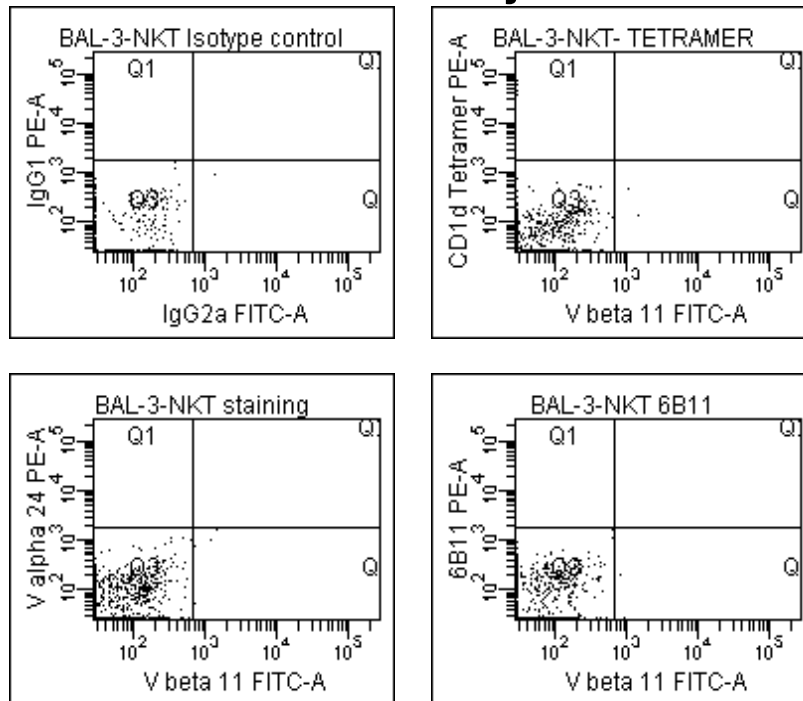
SUP-FIG 11 - Subject 1



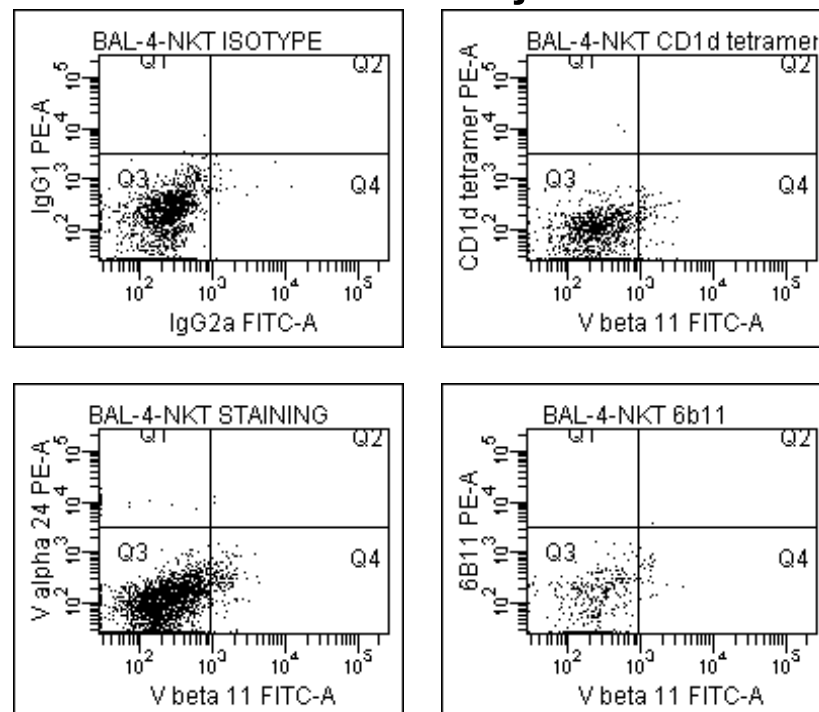
SUP-FIG 11 - Subject 2



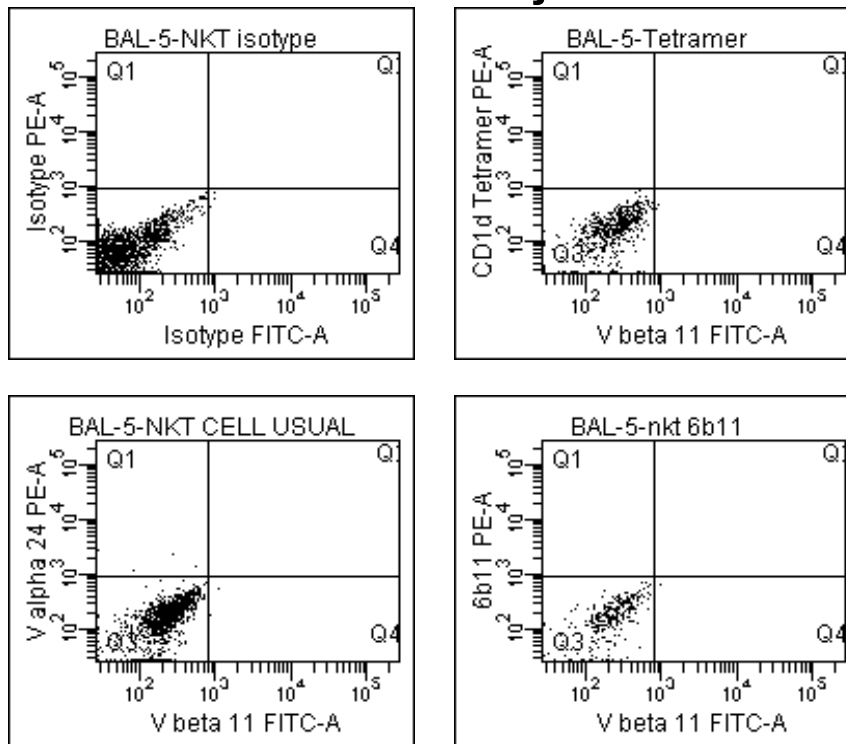
SUP-FIG 11 - Subject 3



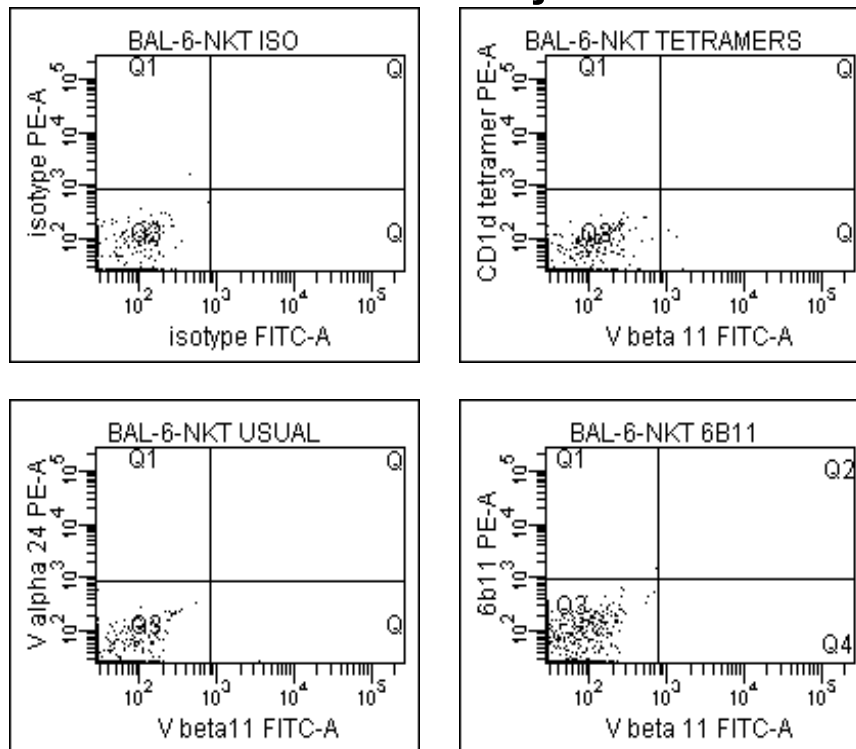
SUP-FIG 11 - Subject 4



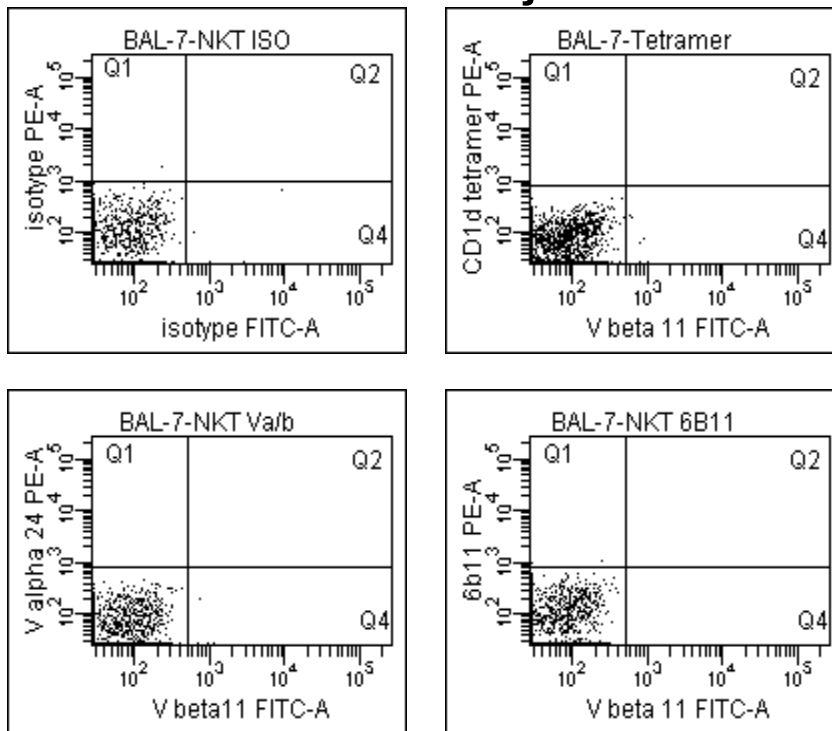
SUP-FIG 11 - Subject 5



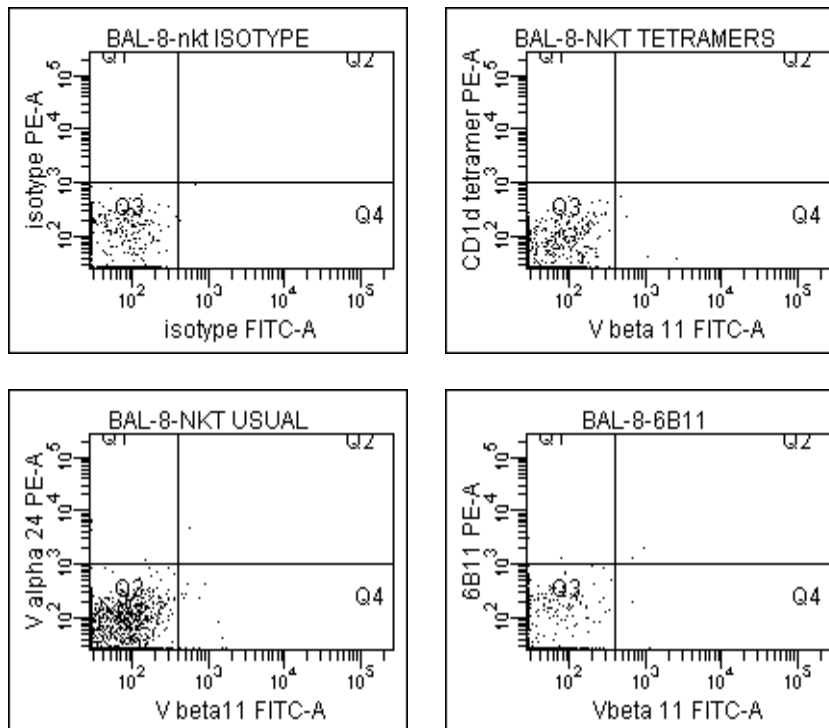
SUP-FIG 11 - Subject 6



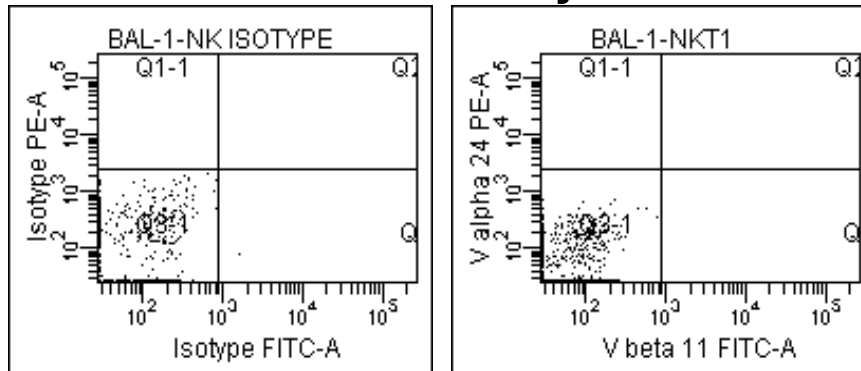
SUP-FIG 11 - Subject 7



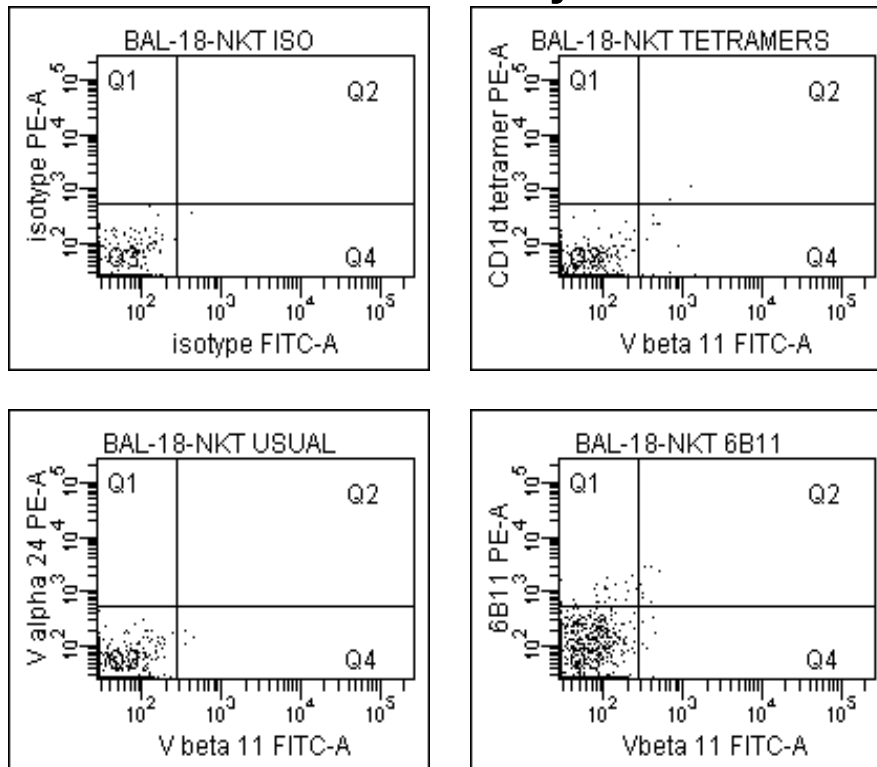
SUP-FIG 11 - Subject 8



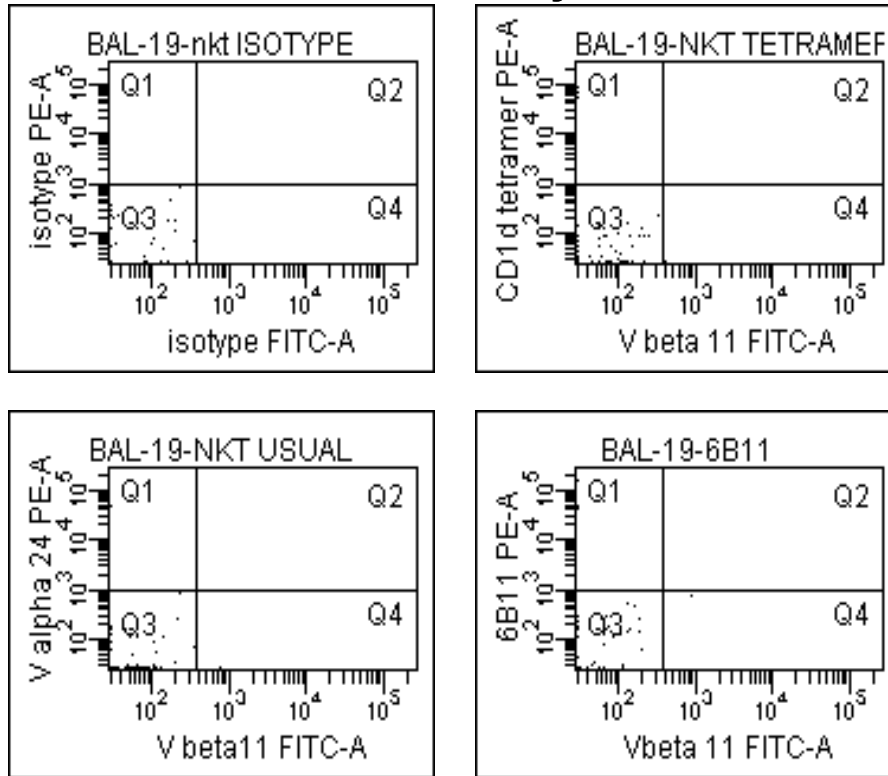
SUP-FIG 11 - Subject 17



SUP-FIG 11 - Subject 18



SUP-FIG 11 - Subject 19



SUP- Figure E11. Individual flow cytometry data plots from asthmatic BAL samples. Staining of BAL samples from asthmatic subjects for iNKT cells, using PE-conjugated CD1d Tetramer+ α Gal and PE-conjugated 6B11 antibody for the CDR3 region of the V α 24 domain in addition to the standard PE-conjugated anti-V α 24 and FITC-conjugated anti-V β 11 antibodies. All the analysis is based on gating on CD3+ T cells. Percentages of CD3+ T cells which are either double positive for V α 24 and V β 11+, or single positive CD1d Tetramer+ α Gal or 6B11 mAb are shown.

Reference List

1. Panina-Bordignon P, Papi A, Mariani M et al. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J Clin Invest* 2001; 107(11):1357-1364.
2. Gunther U, Holloway JA, Gordon JG et al. Phenotypic Characterization of CD3-7+ Cells in Developing Human Intestine and an Analysis of Their Ability to Differentiate into T Cells. *J Immunol* 2005; 174(9):5414-5422.
3. Akbari O, Faul JL, Hoyte EG et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N Engl J Med* 2006; 354(11):1117-1129.
4. Karadimitris A, Gadola S, Altamirano M et al. Human CD1d-glycolipid tetramers generated by in vitro oxidative refolding chromatography. *Proc Natl Acad Sci U S A* 2001; 98(6):3294-3298.