

## 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 <sub>1</sub> % of predicted	FEV <sub>1</sub> : 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
<b>Patients with asthma not treated with corticosteroids</b>	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	

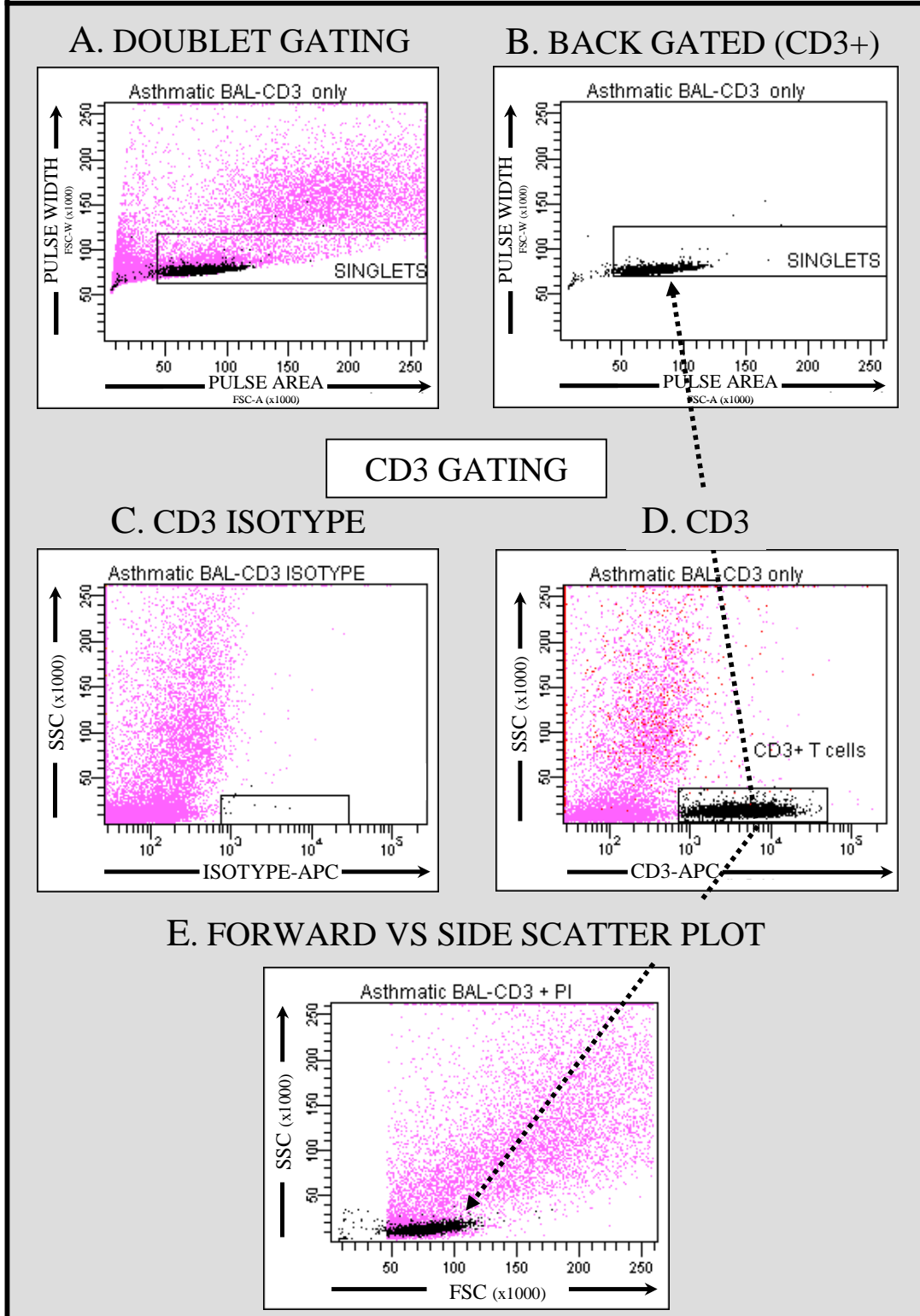
<b>Patients with COPD exacerbation</b>	37	79	M	-	Salbutamol, ipratropium bromide, fluticasone propionate, salmeterol	-	9	0.45
	38	79	F	-	Fluticasone propionate, salmeterol, salbutamol, ipratropium bromide	-	23	0.86
	39	70	F	-	Fluticasone propionate, salmeterol, salbutamol, ipratropium bromide	-	17	0.65
	40	71	M	-	Fluticasone propionate, salmeterol, salbutamol, tiotropium bromide, theophylline	-	18	0.77
	41	48	F	-	Beclomethasone diopropionate salbutamol, ipratropium bromide	-	15	0.68
<b>Patients with stable COPD</b>	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.**

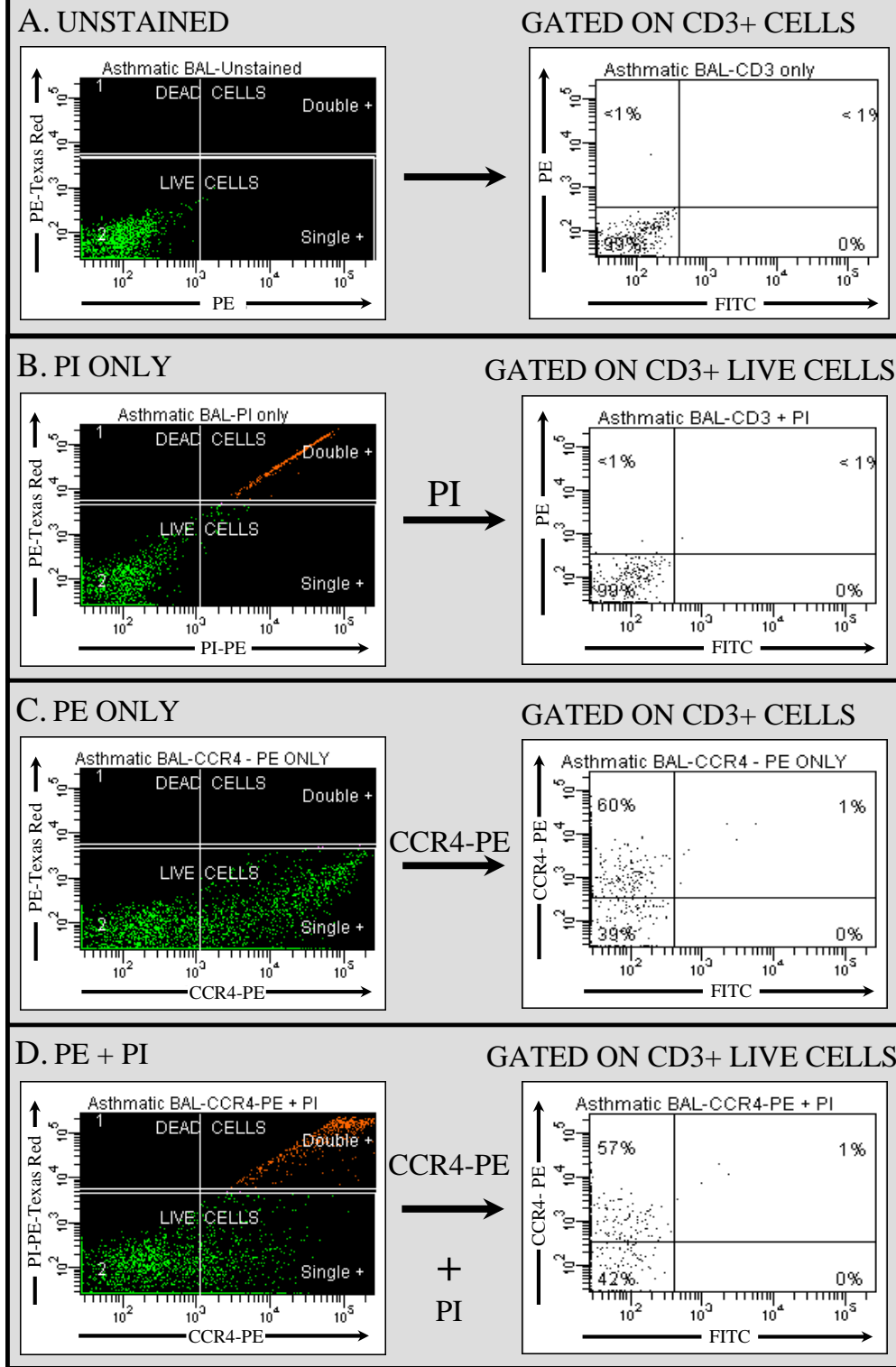
Initially, cell doublets, which can contain adherent cells (e.g. macrophages) or dead cells, and thus bind antibodies non-specifically, are gated out using pulse width and area (SUP-figure E1A) (also see figure 1B in main text) by excluding cells with area less than  $50 \times 10^3$  and pulse width greater than  $120 \times 10^3$ . 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<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> cells with low SSC excludes lymphocytes which contain the iNKT population.

## SUP-FIG E1. GATING STRATEGY FOR DOUBLETS & CD3+ CELLS



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

## SUP-FIG E2. GATING STRATEGY FOR EXCLUDING DEAD CELLS

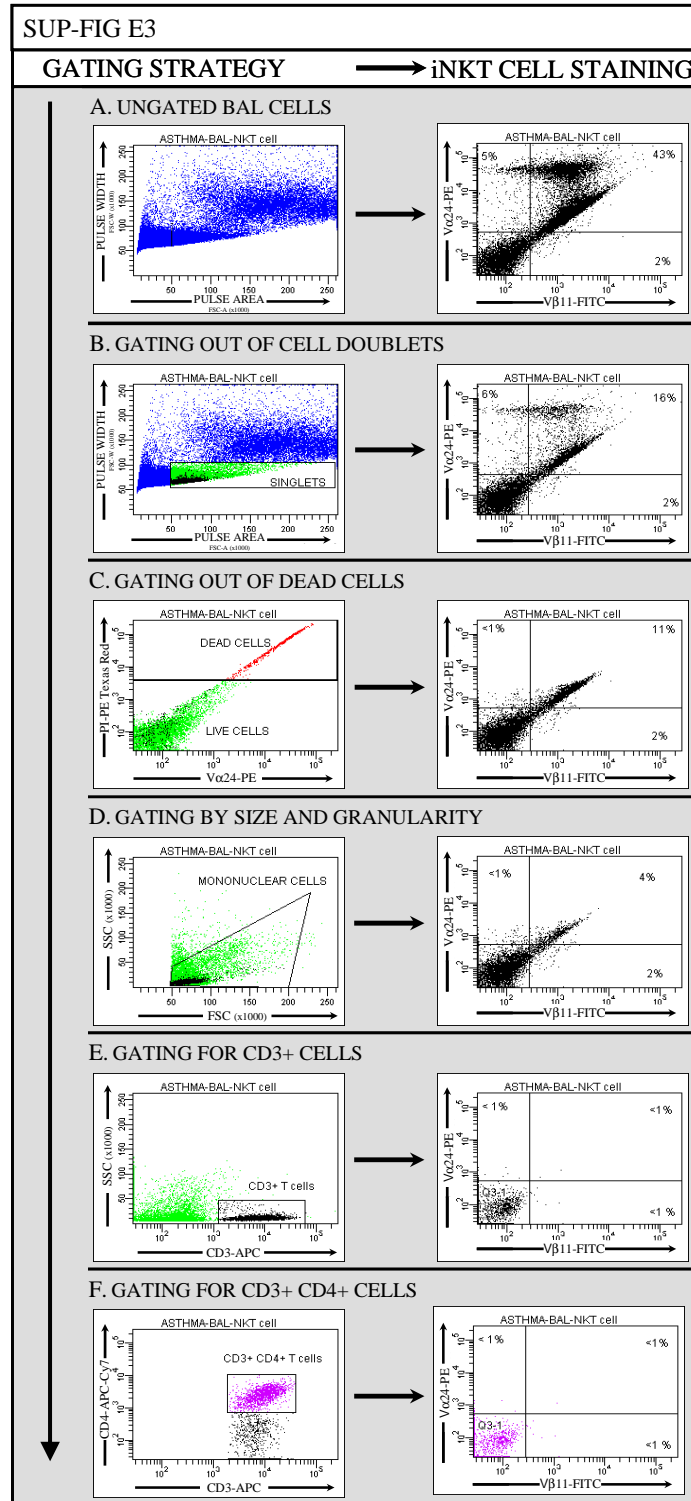


SUP-Figure E2. Gating strategy for excluding dead cells.

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<sup>+</sup>/PE-Texas Red<sup>+</sup>. Only these double positive cells are removed from further analysis while retaining the PE<sup>+</sup>/PE-Texas Red<sup>-</sup> live fraction. When using PE-conjugated anti-CCR4 antibody (C-C chemokine receptor 4 (CCR4) is expressed predominantly in Th2 T cells in asthmatic lungs<sup>1</sup>), 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<sup>+</sup>/PE-Texas Red<sup>+</sup> 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+ $\alpha$ Gal (figure 1 in the article), when stringent gating is not applied, erroneously high percentages of iNKT cells can be detected.



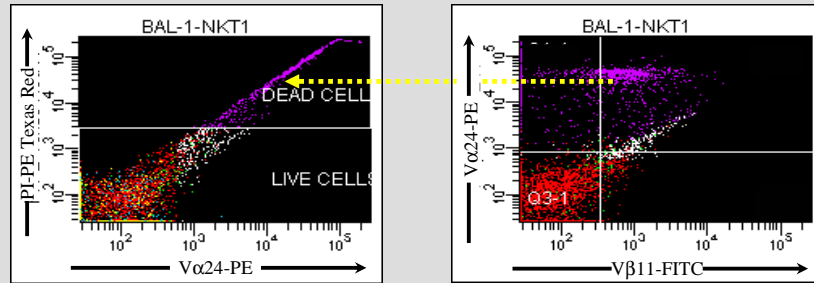
SUP-Figure E3: BAL cell analysis for the surface expression of the invariant NKT T cell receptor (TCR). BAL cells were separated from the fluid phase and incubated in the presence of antibodies for CD3 (APC-conjugated), CD4 (APC-Cy7-conjugated) and the invariant TCR of iNKT cells (V $\alpha$ 24 and V $\beta$ 11) (PE- and FITC-conjugated, respectively) and analysed by 7-colour flow cytometer (FACSAria<sup>TM</sup>). 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<sup>+</sup> 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**

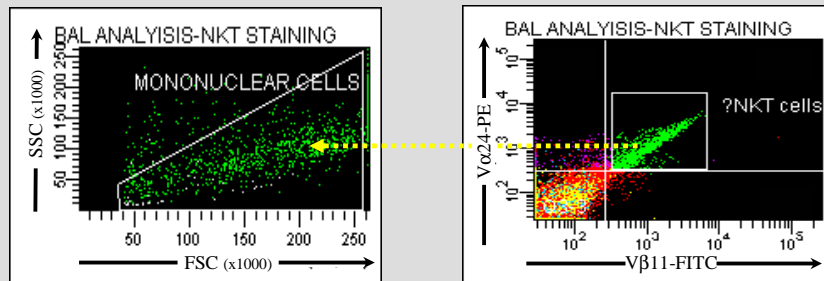
Analysis of BAL cells by flow cytometry is made difficult by the presence of auto-fluorescent alveolar macrophages which are present in large numbers in the recovered BAL fluid. Autofluorescent macrophages can also bind non-specifically both the antibodies and the CD1d Tetramer+ $\alpha$ Gal used for 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 $\gamma$  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 $\gamma$  receptor. In SUP-figure E4C we show that, unless gating is done on scatter properties, high numbers of CD4<sup>+</sup> cells that stain for V $\alpha$ 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<sup>+</sup> 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  $\alpha$ -galactosylceramide unless stringent gating is followed as shown in SUP-figures E5 and E6.

## SUP-FIG E4. FALSE POSITIVE DETECTION OF iNKT CELLS

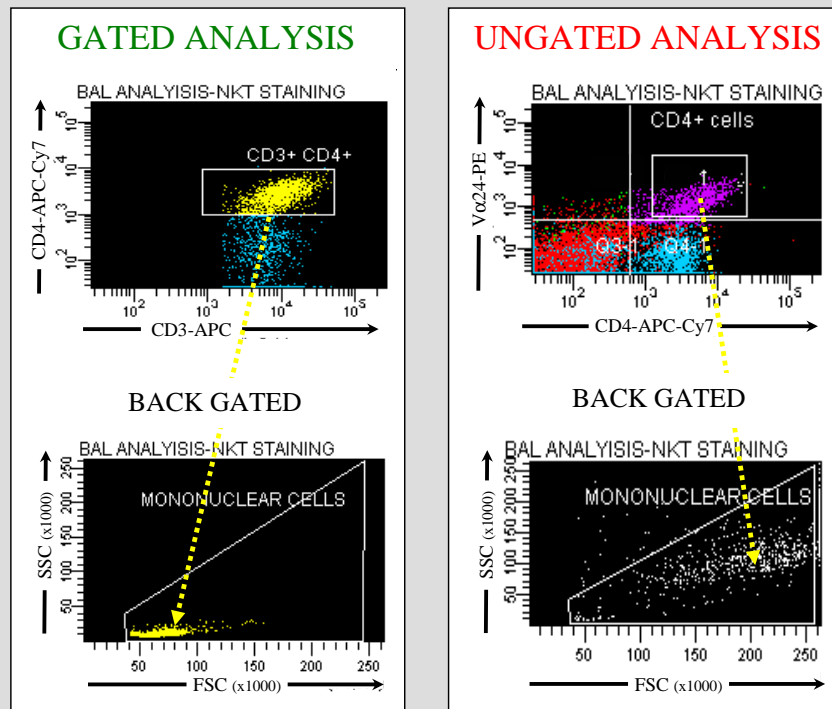
### A. DEAD CELLS STAIN WITH iNKT ANTIBODIES



### B. MACROPHAGE AUTOFLUORESCENCE



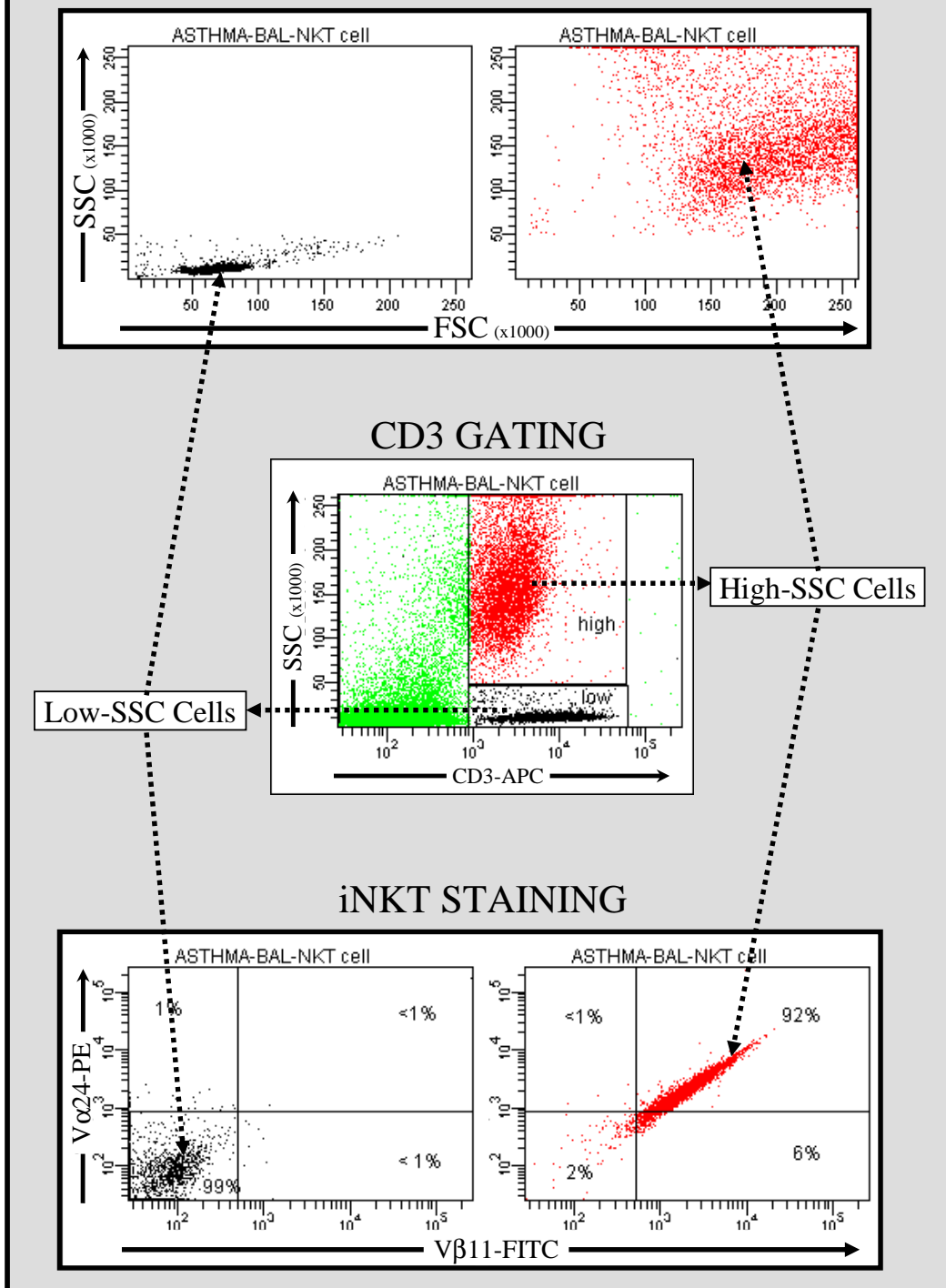
### C. FALSE POSITIVE NKT CELLS IN CD4+ CELLS



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

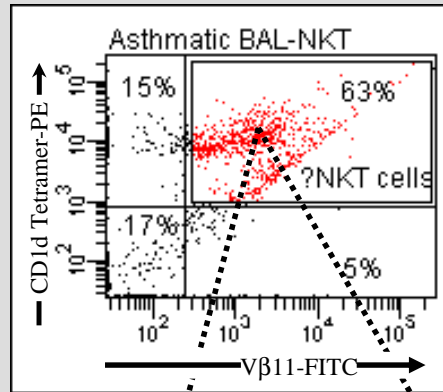
BACK GATING IN FORWARD  
vs SIDE SCATTER PLOT



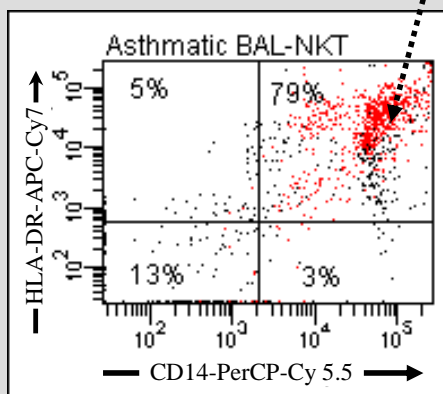
SUP-Figure E5. False positive iNKT detection seen with high side-scatter (SSC) CD3+ cells. CD3 gating of BAL cells from an asthmatic subject based on SSC shows two distinct populations: one with a low SSC (shown as black dots) and the other with a high SSC (shown as red dots – see middle plot). Back-gating 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

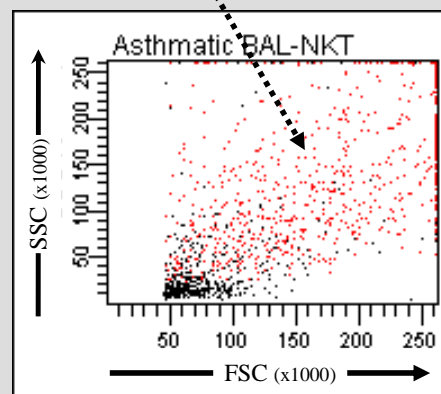
### A. CD1d Tetramer + $\alpha$ GAL & V $\beta$ 11



### B. CD14 & HLA-DR



### C. FORWARD vs SIDE SCATTER PLOT

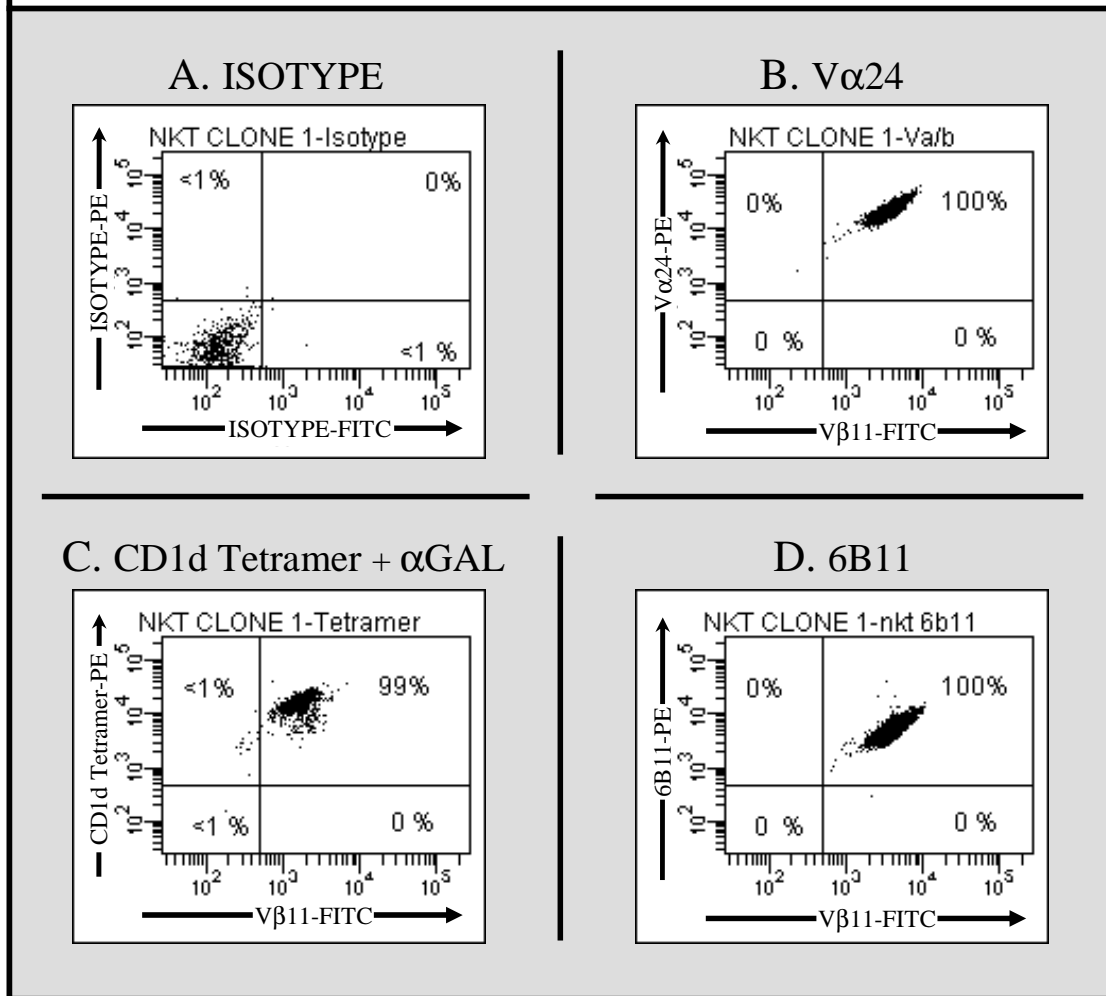


SUP-Figure E6. False positive iNKT cell detection by CD1d Tetramer+ $\alpha$ Gal. When gating procedures, as described in figure 1, are not applied, a significant fraction of cells in BAL samples are stained with CD1d Tetramer+ $\alpha$ Gal and also other antibodies (antiV $\beta$ 11) (A). However, back-gating of these CD1d Tetramer+ $\alpha$ 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 $\alpha$ 24 and V $\beta$ 11 T cell receptor on iNKT cells. The double positive cells (iNKT cells) were sorted using FACSAria<sup>TM</sup> (BD Biosciences, Oxford, U.K) at 1 cell/well into a 96-well plate containing  $2 \times 10^4$  autologous irradiated (2500 RAD) PBMC. Viable iNKT cells were expanded in the presence of phytohaemagglutinin (PHA-p Sigma, Poole, U.K) at 1  $\mu$ 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 $\alpha$ 24, anti-V $\beta$ 11, anti-6B11 antibodies and CD1d Tetramer+ $\alpha$ Gal (SUP figure E7).

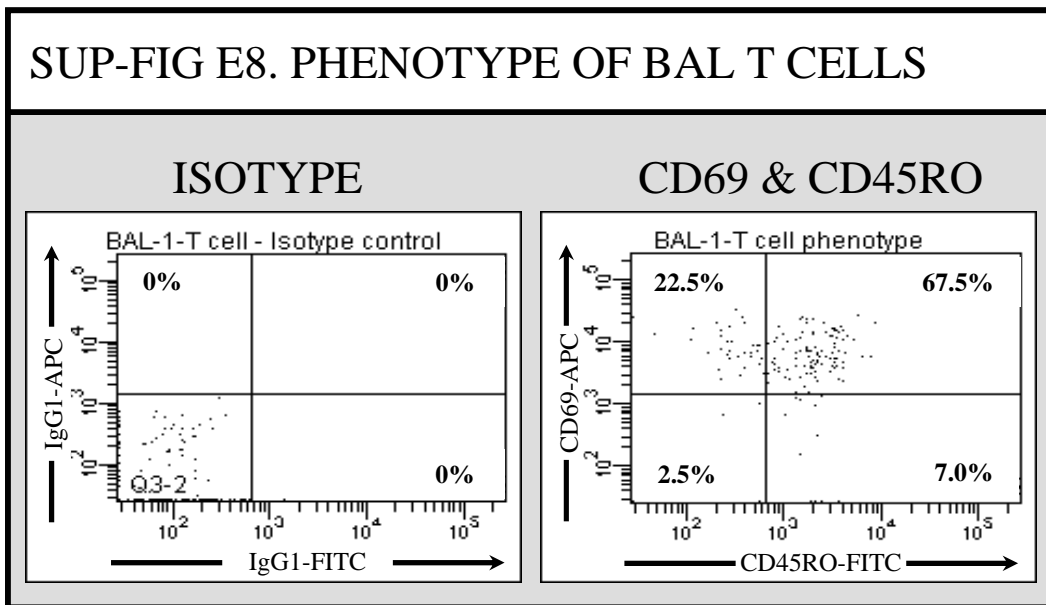
## SUP-FIG E7. VALIDATION OF iNKT CELL CLONE



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 V $\alpha$ 24 and V $\beta$ 11 domains of the TCR (B). Similar uniform staining is also shown with 6B11 antibodies (D) and CD1d Tetramer+ $\alpha$ Gal (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.



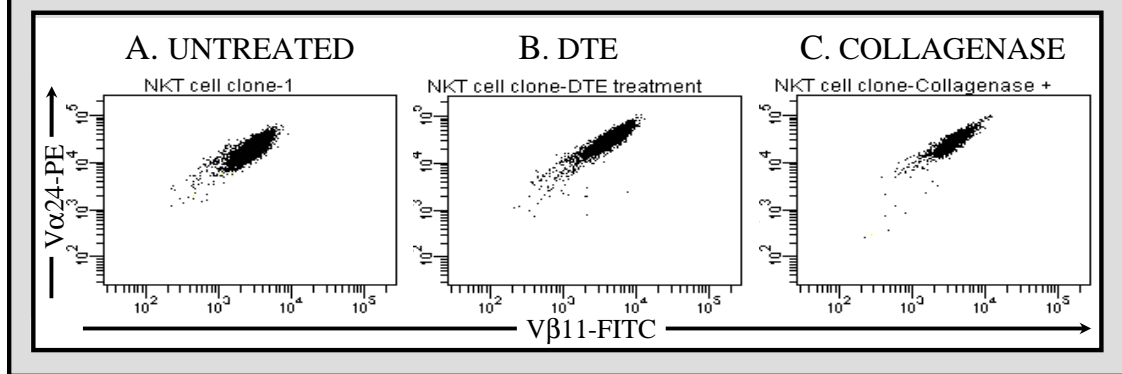
SUP-Figure E8. Flow cytometric analysis of BAL cells showing that 74.5% of T cells are CD45RO+, indicating their memory phenotype, and that 90% of them are activated, expressing surface CD69. (Abbreviations in the figure IgG1: Immunoglobulin G1)

## **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 $\alpha$ 24 and V $\beta$ 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- $\mu$ 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 $\gamma$  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 treatment<sup>2</sup>, analysis was restricted to CD3<sup>+</sup> T cells. As shown in figure E9C, collagenase treatment of the iNKT cell clone was shown not to affect the detection of either V $\alpha$ 24 or V $\beta$ 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 V $\alpha$ 24 and V $\beta$ 11 domains of the TCR. Surface expression of V $\alpha$ 24 and V $\beta$ 11 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 $\alpha$ 24 (TRAV10 gene):

F- AAGCATCTGACGACCTTCTTG, R-AACAGGACCTCTCCCAGTATC

2. TCR V $\beta$ 11 (TRBV25-1 gene):

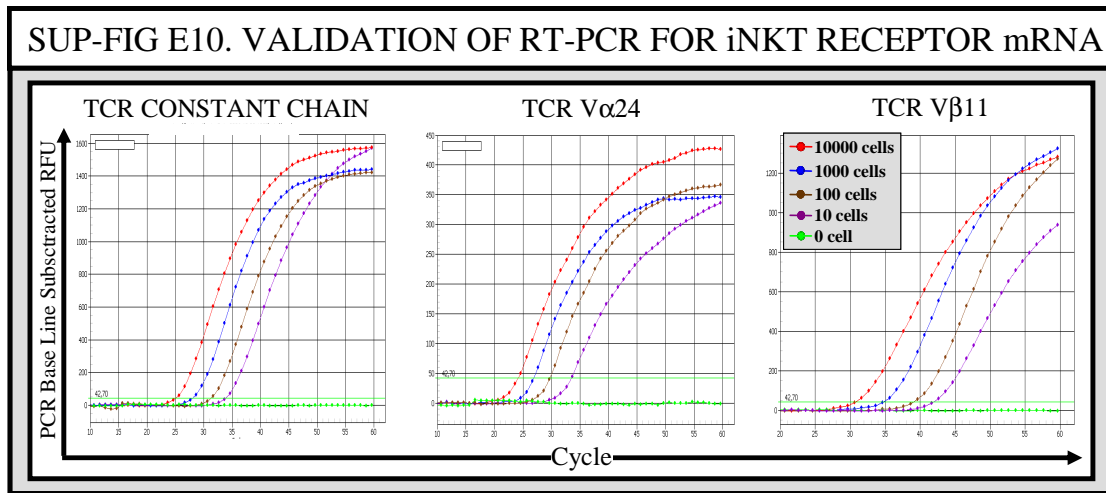
F- GCACAGTTTGGGCTTTTATTTT, R-GAGAACATTCCAGAGTGATCTTC

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 $\alpha$ 24 and V $\beta$ 11). In contrast, the asthmatic BAL cells did not express mRNA for the invariant NKT cell receptor (V $\alpha$ 24 and V $\beta$ 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 $\alpha$ 24, V $\beta$ 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 $\alpha$ 24 and V $\beta$ 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 $\alpha$ 24 and V $\beta$ 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 $\alpha$ 24 and V $\beta$ 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 $\alpha$ 24	V $\beta$ 11
<b>iNKT cell clone</b>		27.6	27.2	29.6
<b>Moderate asthmatic subjects on inhaled corticosteroids</b>	7	28.8	n.d.	n.d.
	15	35.3	n.d.	n.d.
	16	30.1	n.d.	37.1
<b>Mild asthmatic subjects, steroid-naïve</b>	24	40.5	n.d.	n.d.
<b>Healthy subjects</b>	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
<b>COPD subjects</b>	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 $\alpha$ 24 and V $\beta$ 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 $\alpha$ -galactosylceramide**

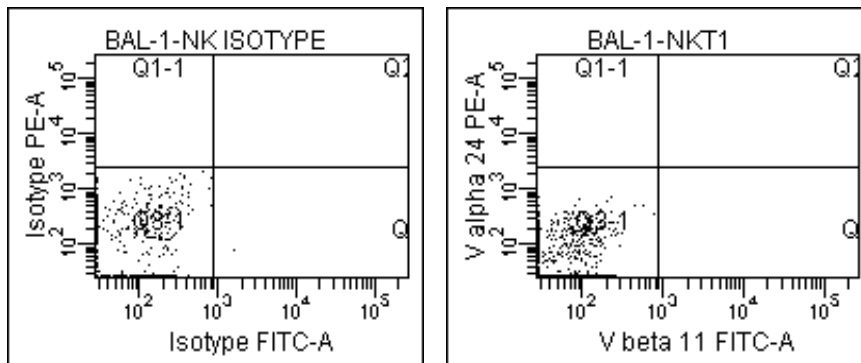
We have attempted to reproduce the *in vitro* findings of Akbari et al.<sup>3</sup> by stimulating BAL cells, obtained by bronchoscopy from 4 asthmatic subjects, with  $\alpha$ -galactosylceramide for a period of 24 hours. Supernatants were assessed for IL-5 and IL-13. Given that no details of time-course 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 ( $2 \times 10^6$ /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  $\alpha$ -galactosylceramide only, 2)  $\alpha$ -galactosylceramide (100 ng/ml) (standard dose used by others<sup>4</sup>, 3)  $\alpha$ -galactosylceramide (500 ng/ml) (higher dose), 4)  $\alpha$ -galactosylceramide (100 ng/ml) + anti-human CD1d blocking antibody (25  $\mu$ g/ml, clone CD1d42, mouse IgG1 kappa (BD Pharmingen<sup>TM</sup>) to ensure CD1d restriction of measured cytokine responses, 5)  $\alpha$ -galactosylceramide (100 ng/ml) + mouse IgG1 kappa isotype control antibody (25  $\mu$ g/ml (BD Pharmingen), 6)  $\beta$ -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  $\mu$ 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

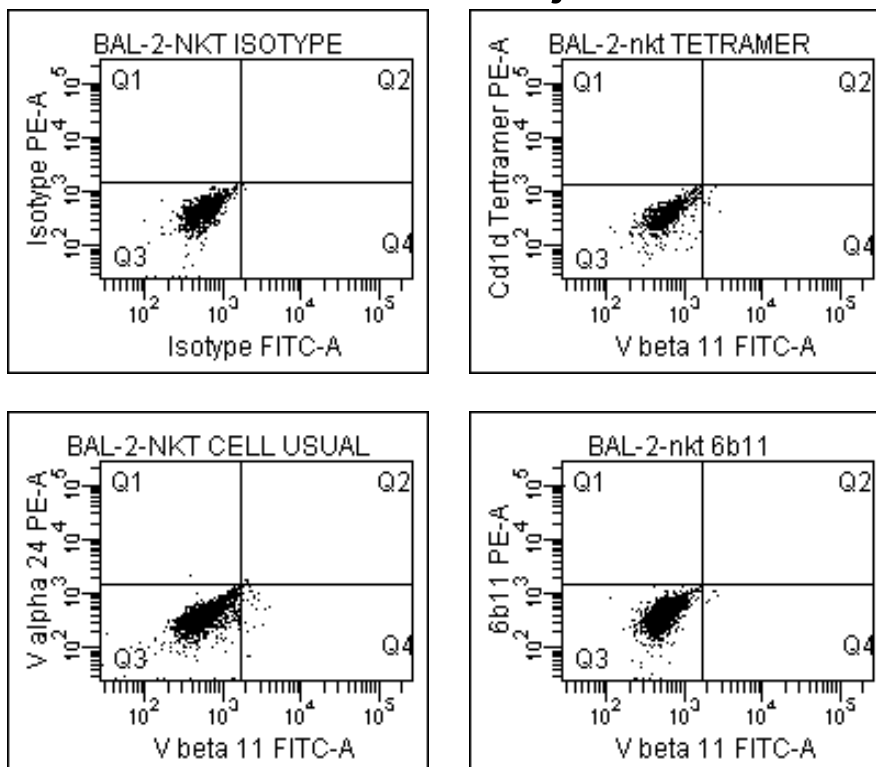
with 1)  $\alpha$ -galactosylceramide, 2) the irrelevant lipid,  $\beta$ -galactosylceramide, 3) house dust mite allergen extract or 4) anti-CD3/CD28 antibody. None of these subjects had detectable iNKT cells in their BAL by flow cytometry.

Flow cytometric analyses using 6B11 and CD1d Tetramer+ $\alpha$ 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 $\alpha$ 24 and V $\beta$ 11 and isotype control (SUP-figure E11).

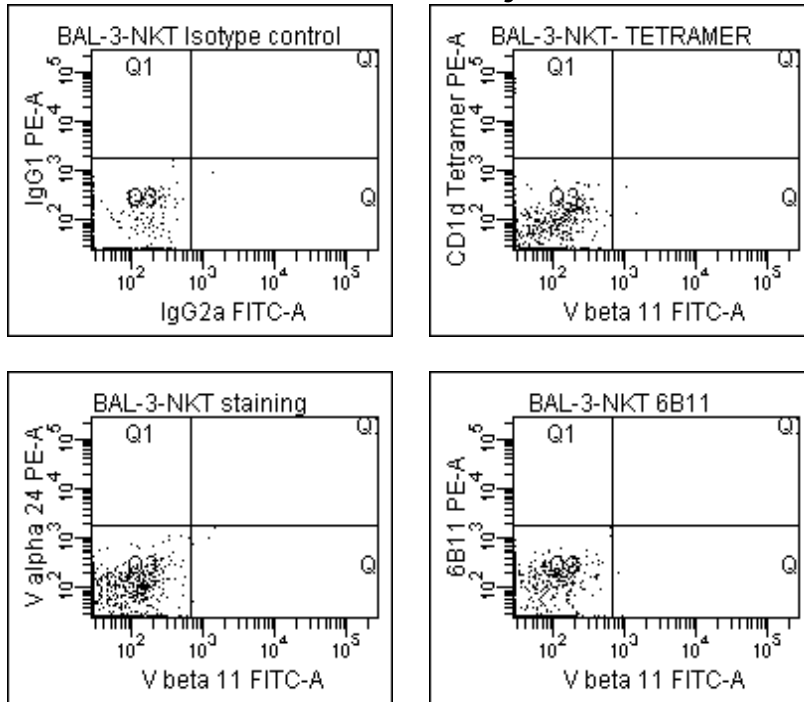
## SUP-FIG 11 - Subject 1



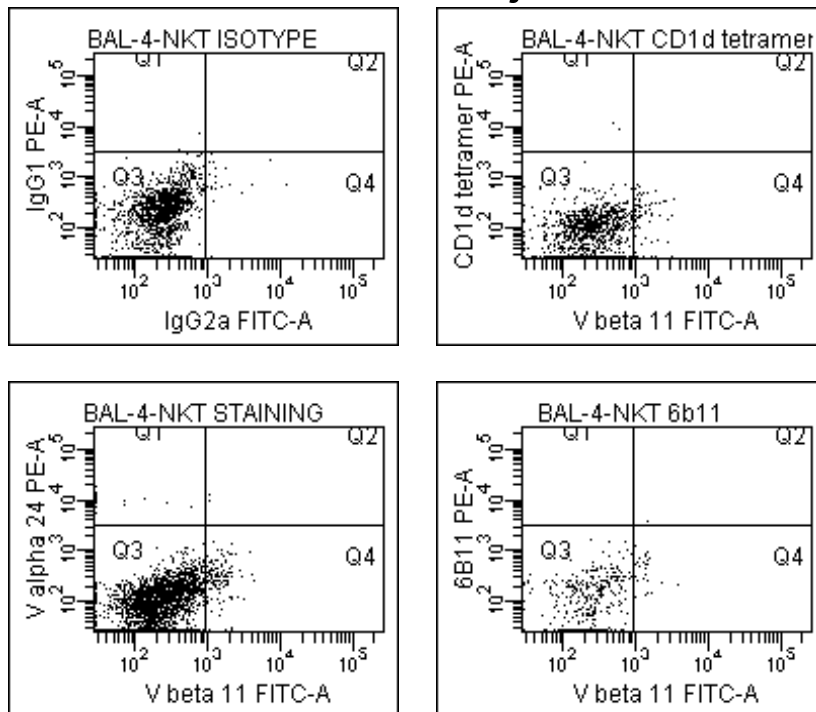
## SUP-FIG 11 - Subject 2



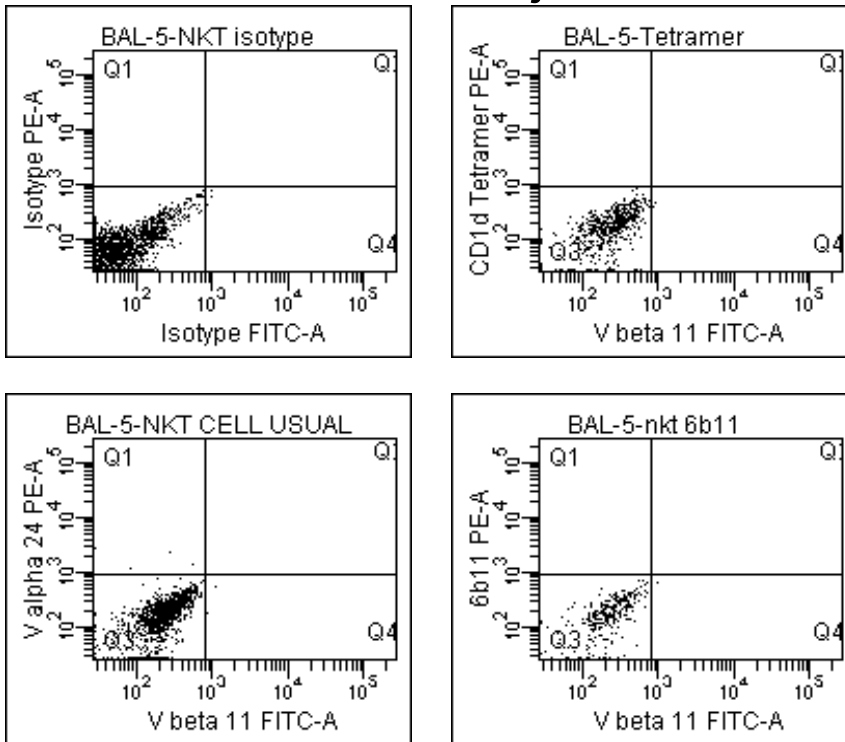
## SUP-FIG 11 - Subject 3



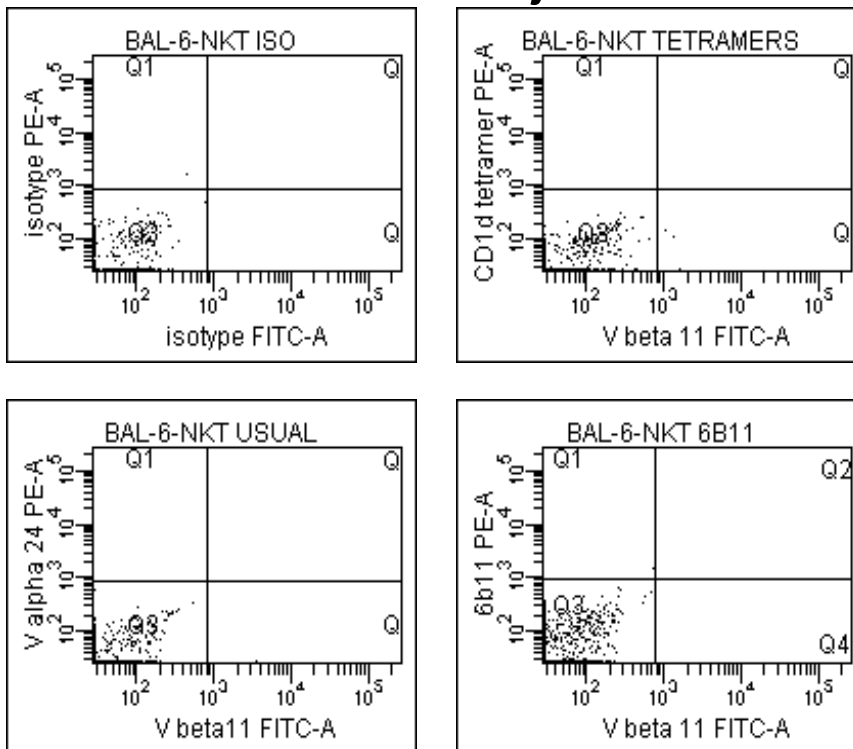
## SUP-FIG 11 - Subject 4



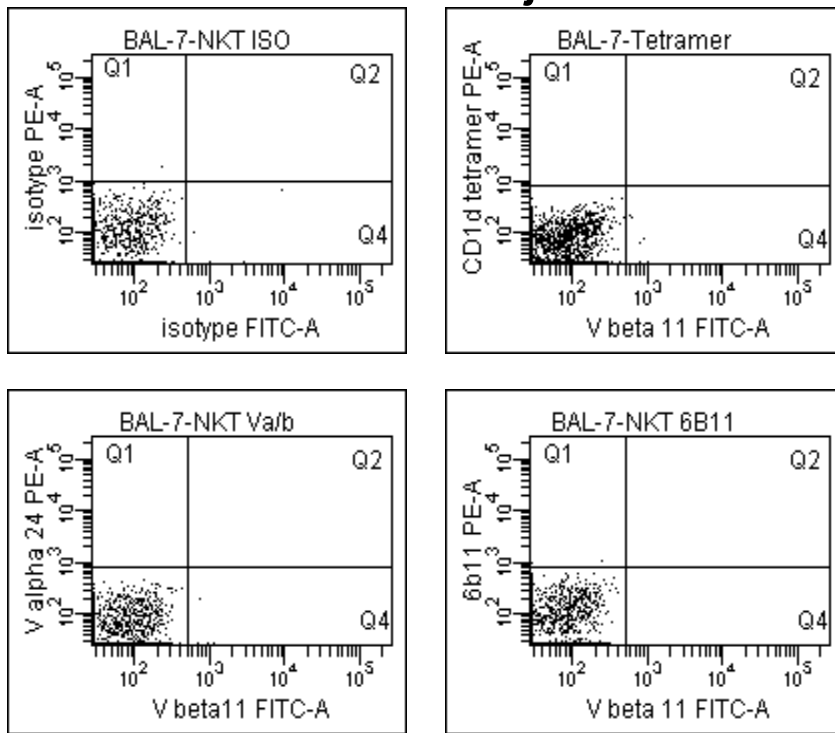
# SUP-FIG 11 - Subject 5



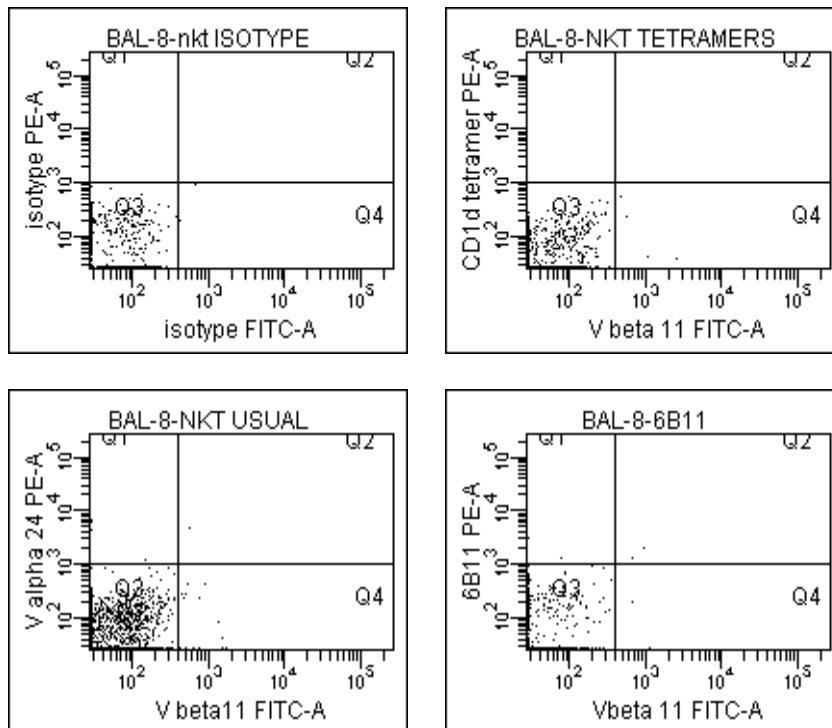
# SUP-FIG 11 - Subject 6



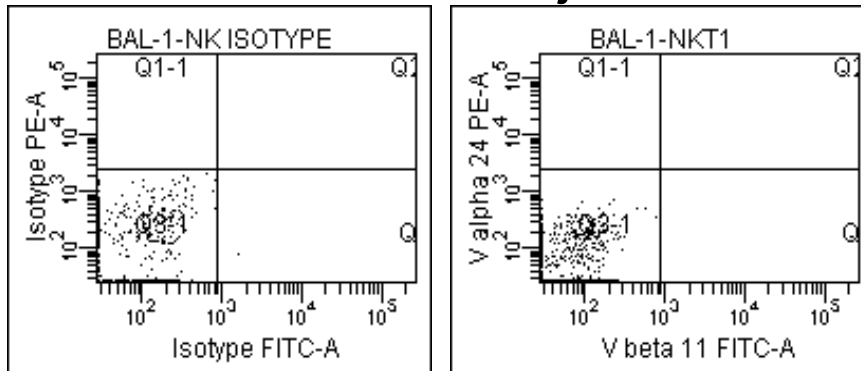
## SUP-FIG 11 - Subject 7



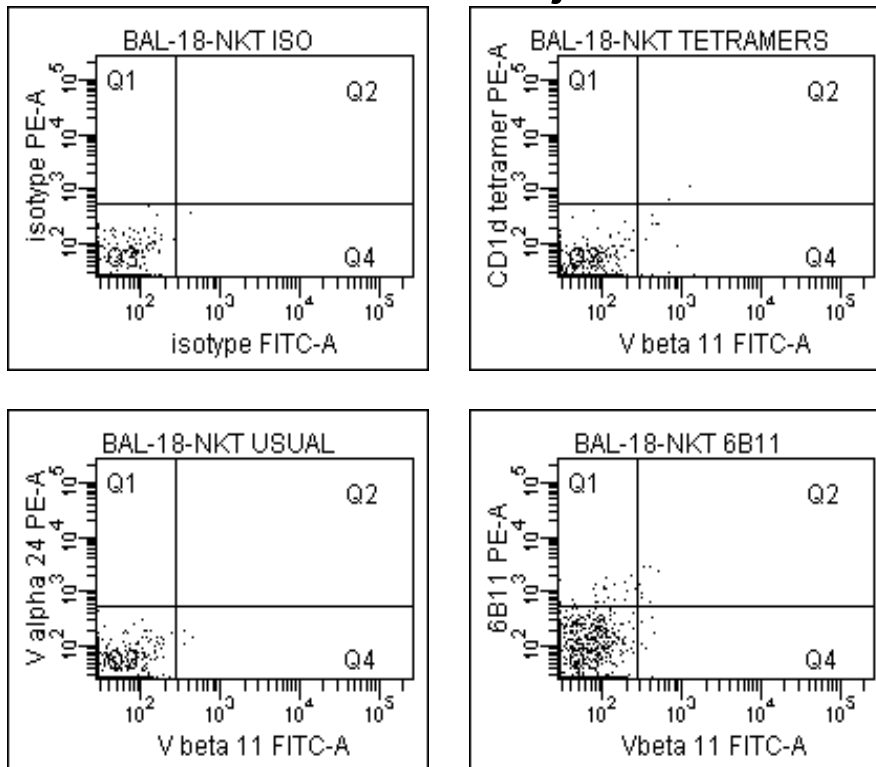
## SUP-FIG 11 - Subject 8



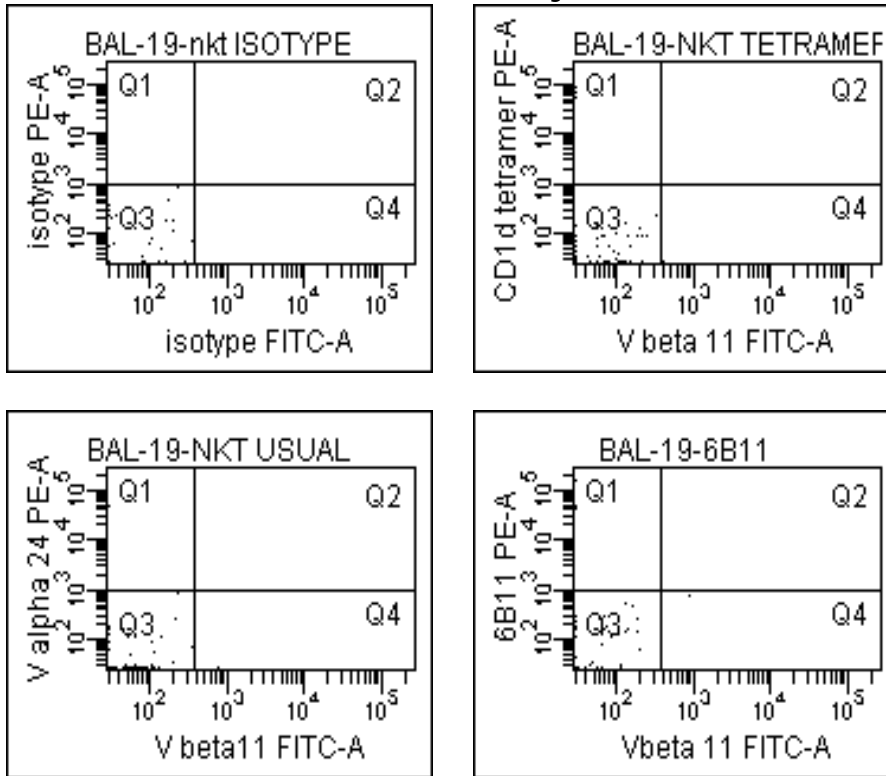
## SUP-FIG 11 - Subject 17



## 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+ $\alpha$ Gal and PE-conjugated 6B11 antibody for the CDR3 region of the V $\alpha$ 24 domain in addition to the standard PE-conjugated anti-V $\alpha$ 24 and FITC-conjugated anti-V $\beta$ 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 $\alpha$ 24 and V $\beta$ 11+, or single positive CD1d Tetramer+ $\alpha$ Gal or 6B11 mAb are shown.

## Reference List

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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.