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Natural Killer T Cells in the Lungs of Patients with Asthma

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To editor

Based on studies in mice, an important role for invariant Natural Killer T (iNKT) cells in the pathogenesis of asthma has been proposed^{1–4}. However, the role of iNKT cells in human asthma is controversial, because there is disagreement on whether iNKT cells are present in the lungs of patients with asthma^{5, 6}. To address this controversy, we examined bronchoalveolar lavage fluid (BALF) from asthmatics with a broad range of severity and symptom control.

The study was approved by the institutional review boards at Children's Hospital Boston, Brigham and Women's and Connolly Hospitals. All 18 subjects provided written informed consent and underwent fiberoptic bronchoscopy. Five had severe asthma (based on the NHLBI Severe Asthma Research Program (SARP) criteria)⁷, eight had well-controlled asthma (based on the NHLBI-EPR3 Guidelines) and five had no history of asthma (Table 1). BALF samples were analyzed for the presence of iNKT cells by flow cytometry (8-Color BD FACSCanto, FlowJo software), by investigators who were blinded from clinical data at the time of the analysis. BALF cells were filtered through 70 µm cell strainers to remove mucous, and

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examined for viability with trypan blue (mean viability 95%) before treatment with human IgG to block Fc receptors. Cells were then stained with PBS57 (analog of α -GalCer)-loaded CD1d tetramer-PE (or unloaded CD1d tetramer-PE control) (NIH tetramer facility) and anti-CD3-Alexa750 (BD bioscience). We gated on the CD3⁺ low side scatter population to exclude alveolar macrophages, which have high side scatter and high auto-fluorescence (Figure 1a). The percentage *i*NKT cells of total T cells was then analyzed (PBS57 loaded CD1d tetramer⁺CD3⁺ cells/total CD3⁺ cells). Background staining (unloaded CD1d tetramer⁺CD3⁺/CD3⁺) was then subtracted (Figure 1a, b) to calculate adjusted percentage *i*NKT cells of total T cells for statistical analysis (Figure 2).

We found that patients with severe asthma had a significant increase in the number of BALF *i*NKT cells when compared to that of non-asthmatic controls ($p=0.004$ Mann-Whitney test) (Figure 2). Some but not all patients with well-controlled asthma had an increase in the number of BALF *i*NKT cells compared to non-asthmatic controls ($p=0.02$). We conclude that *i*NKT cells are present in the BALF of some but not all patients with asthma. The specific number of *i*NKT cells present in the BALF was quite variable, but patients with severe asthma appeared to more consistently have an increase in BALF *i*NKT cells than patients with well-controlled asthma, the majority of whom did not have an increase in the number of BALF *i*NKT cells over that of non-asthmatic individuals.

These results suggest that asthma severity and control might be loosely related to the number of pulmonary *i*NKT cells. However, it is also likely that many other factors are involved in determining the number of pulmonary *i*NKT cells. The number of *i*NKT cells in the BALF appears to be extremely dynamic and fluctuates rapidly (in mice, *i*NKT in BAL fluid range from 1% of lung lymphocytes in naïve mice to 14% after allergen challenge). We do not yet fully understand the factors that regulate the number of *i*NKT cells in the lung. In our previous study of poorly controlled asthmatics, all of asthmatic patients had higher numbers of pulmonary *i*NKT cells compared to that in patients with sarcoidosis or normal individuals, but the specific number of *i*NKT cells present was much higher than in the current study. This difference in the number of pulmonary *i*NKT cells could be due to different patient selection criteria and possibly to improved methods for analyzing *i*NKT cells.

Importantly, our current results help to explain the results observed in the previous ten studies of *i*NKT cells in human asthma. In seven of the studies a significant increase in the number of *i*NKT cells in the lungs of patients with asthma compared to that in the normal individuals was observed^{4, 5}(see supplemental reference list in the online repository). In an eighth study, there was a trend toward an increase in the *i*NKT cells in asthma above that of control subjects, but this did not reach statistical significance⁸. In two additional studies^{6, 9}, several asthmatic patients were observed to have an increase in the number of *i*NKT cells in BALF (up to 2.1–2.7% of BALF lymphocytes). However, most of the patients had much fewer pulmonary *i*NKT cells, such that the authors concluded that *i*NKT cells were not important in the pathogenesis of asthma, although BALF from normal individuals was not examined^{6, 9}. In our current study of patients with a very broad range of asthma severity and control, we observed an increase in the number of pulmonary *i*NKT cells in all five patients with severe asthma, and in some but not all of the patients with well controlled asthma, consistent with all ten previous studies taken together.

While addressing the major and controversial question of whether *i*NKT cells are present in the lungs of asthmatics, our study did not focus on other important issues regarding *i*NKT cells, including whether the number of *i*NKT cells in the lung varies over time or with exacerbations, analysis of the phenotype and the subsets of *i*NKT cells present in the lungs and whether distinct subsets of *i*NKT cells are present in different forms of asthma, as suggested by murine studies^{1–4}. The limitations of our study were due in part to the small number of patients

included in our report, and the fact that repeat bronchoscopy of patients with severe asthma cannot be easily accomplished. However, our results strongly suggest that additional studies with a larger number of subjects are warranted to examine these questions.

The observation that *i*NKT cells are present in some patients with asthma suggests that *i*NKT cells may contribute to the pathogenesis of asthma. This question is of much greater importance but must be addressed by examination of *i*NKT cell function in asthma. Such functional studies are difficult to perform in humans for ethical and safety reasons, but studies in mice and in non-human primates provide a very compelling argument regarding the importance of *i*NKT cells in the development of AHR. Thus, in three distinct models of asthma in mice, induced with allergen^{1, 2}, virus⁴, or by exposure to ozone³ (a component of air pollution), AHR failed to develop in the absence of *i*NKT cells. In each of these models, a different subset of *i*NKT cell was required for AHR. Moreover, functional studies of *i*NKT cells, performed by direct and specific activation of *i*NKT cells in naïve mice¹⁰ or in cynomolgus monkeys¹¹ resulted in the development of AHR, suggesting that even the few *i*NKT cells present in the normal lung, when directly activated can result in AHR. These functional studies of *i*NKT cells together suggest that *i*NKT cells may represent an important common element that links many different forms of asthma, including allergic and non-allergic asthma. These results also suggest that the absolute number of *i*NKT cells present in the lungs may not be as important as their functional capacity when activated to contribute to the development of AHR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

<i>i</i>NKT cells	invariant Natural Killer T cells
AHR	airway hyperreactivity
TCR	T cell receptor
BALF	Bronchoalveolar lavage fluid
FEV1	forced expiratory volume in 1 st second

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Figure 1.

a) *i*NKT cells in BALF. We gated on CD3⁺ low side-scatter cells (T-cells), and then analyzed for expression of PBS57-loaded CD1d-tetramer (Y-axis) versus CD3 (X-axis) (*i*NKT, right panel of each pair), or for expression of unloaded CD1d-tetramer (Y-axis) versus CD3 (X-axis) (background, left panel of each pair).

b) *i*NKT-cells and background staining for each subject, categorized by asthma severity/symptom control.

Figure 2. Adjusted % *i*NKT cells/T cells were calculated by subtracting background from % *i*NKT/T cells. Statistical comparison of adjusted % *i*NKT/T cells in BALF between poorly controlled or well controlled asthma and non-asthmatic control using Mann-Whitney test.

Table 1

Subjects' demographic and clinical information

Group	Subject ID	Gender	Age (year)	%FEV1 [*]	Exacerbation [#]	Duration (year) ⁺	ICS dose [§]	Other medication [¶]	Allergies ^ψ	Concomitant disease
Severe asthma	P1	Female	50	78	1	50	1000	salmeterol, montelukast	cockroach	hypertension
	P2	Male	31	56	2	25	1000	salmeterol tiotropium montelukast	none	none
	P3	Male	50	41	2	50	1000	salmeterol theophylline	cockroach	diabetes hypertension
	P4	Male	21	58	1	15	1000	salmeterol	cockroach, cat, mite	none
	P5	Male	49	73	1	40	1880	salmeterol montelukast	Alternaria, tree, Dog, Cat	diabetes hypertension
Mean				61	1.4	36	1176			
Well controlled asthma	W1	Female	35	87	0	25	500	salmeterol	none	none
	W2	Male	41	109	0	20	500	salmeterol	none	none
	W3	Female	22	90	0	3	500	salmeterol	cat	none
	W4	Male	33	103	0	30	500	salmeterol	cockroach	none
	W5	Female	47	86	0	40	440	none	cockroach, rat, mite	none
	W6	Female	56	83	1	3	800	montelukast	none	none
	W7	Female	59	82	1	1.5	264	none	none	hypertension ischemic heart disease
	W8	Female	47	99	0	2	0	none	ND	peptic ulcer dilated cardiomyopathy
Mean				92	0.25	16	438			
No asthma	H1	Male	43	ND	0	0	0	none	ND	sarcoidosis
	H2	Male	39	ND	0	0	0	none	ND	bronchiectasis
	H3	Male	49	99	0	0	0	none	ND	peptic ulcer
	H4	Female	31	114	0	0	0	none	ND	sarcoidosis
	H5	Female	50	89	0	0	0	none	none	none
Mean				107	0	0	0			

^{*} % predicted Force expiratory volume in 1st second at the time of study entry

[#] number of acute asthma exacerbation requiring oral corticosteroid, emergency room or urgent care visit in the past 12 months

⁺ duration that the subject has been diagnosed with asthma

$\S$ inhaled corticosteroid dose (fluticasone or fluticasone equivalent, in ug/day). Medications were not changed for at least 4 weeks prior to bronchoscopy.

$\P$ other medication used for asthma control (not including rescuer medication)

Ψ positive result from allergen skin prick testing.

ND=not done

Six other BALF samples were evaluated using a different methodology. These samples could not be directly compared with the final 18 samples, since the sensitivity for detecting NKT cells was not comparable. The trend of the results with the six other samples was similar to what is reported for the 18 samples.