

## Letter to the Editor

**Bronchoalveolar lavage invariant natural killer T cells are not increased in asthma**

To the Editor:

Asthma is characterized by variable airflow obstruction and airway hyperresponsiveness (AHR) in association with chronic inflammation. The airway inflammation in asthma is typically eosinophilic with increased expression of  $T_H2$  cytokines<sup>1</sup> and mast cell infiltration of the airway smooth muscle bundle.<sup>2</sup> Emerging evidence also supports a possible role in the pathophysiology of asthma for natural killer (NK) T cells and, in particular, CDd1 restricted or invariant NKT (iNKT) cells. NKT cells are a recently identified distinct subset of T cells that express markers of both NK cells and T cells and seem to be potent immunoregulatory cells because of their ability to secrete large quantities of both  $T_H1$  and  $T_H2$  cytokines.<sup>3</sup> In an animal model of asthma, NKT cell-deficient mice were shown to be unable to develop AHR after allergen sensitization and challenge although these animals did show  $T_H2$  responses after the allergen challenge.<sup>4</sup> Subsequent adoptive transfer of NKT cells into these mice restored AHR, and this was shown to occur via an IL-4-dependent and an IL-13-dependent pathway. Furthermore, this transfer seemingly occurs independent of eosinophils, B cells, and  $CD4^+$  T cells, which indicates an additional effector

role for these cells in this model of asthma.<sup>5</sup> In a human study of moderate-to-severe patients with asthma, up to 60% of the  $CD4$  T cells in bronchoalveolar lavage (BAL) were identified as iNKT cells. In addition, these pulmonary iNKT cells were shown to produce exclusively  $T_H2$  cytokines.<sup>6</sup> The scale of this finding is surprising, and given the differing properties of these cells from the conventional  $CD4$  T cells, this does have important implications in understanding the pathophysiology of asthma, especially steroid-resistant asthma, and the development of future treatment strategies. We have therefore enumerated the proportion of BAL NKT cells in subjects with asthma across severities and controls.

Eleven patients with asthma and 5 controls were recruited. All patients with asthma and 3 controls were recruited from Glenfield Hospital in Leicester, United Kingdom, and 2 additional controls were recruited from Queen Elizabeth Hospital Birmingham, United Kingdom (UK). Asthma was defined according to Global Initiative for Asthma (GINA) guidelines, and severity was classified according to GINA treatment steps and the American Thoracic Society (ATS) criteria for severe refractory asthma.<sup>7</sup> The controls included 4 subjects with sarcoidosis and 1 subject with nonasthmatic eosinophilic bronchitis.<sup>2</sup> The study was approved by the local ethics committee, and informed consent was obtained from all subjects.

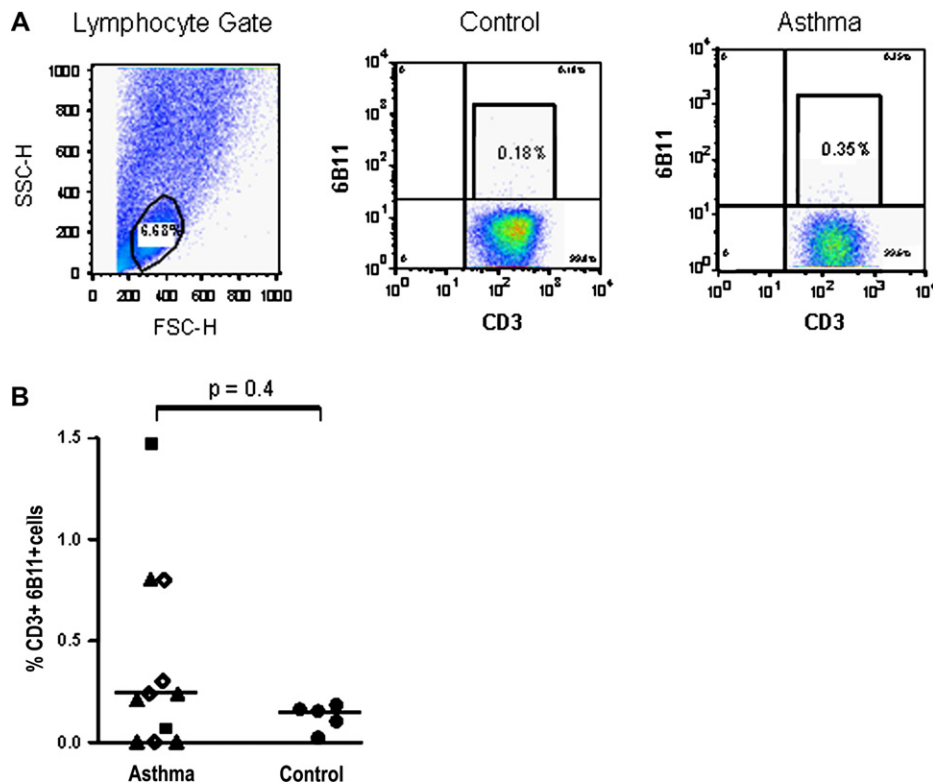


**FIG 1.** A, Representative scatter plots of BAL cells show gating of lymphocytes and fluorescence-associated cell sorting plots of 6B11+ cells within gated CD3 lymphocytes in asthma and control subjects. B, Percentages of 6B11+ NKT cells in asthma [mild (■), moderate (◇), and severe refractory (▲)] and control (●) subjects. Each median percentage is represented by a horizontal bar. P values were calculated using the Mann-Whitney U test.

All subjects underwent bronchoscopy and BAL. BAL was filtered through 48  $\mu$ m nylon gauze to remove debris, and cells were stained with conjugated monoclonal antibodies to CD3 (Caltag-MedSystems Ltd, Buckingham, UK) and 6B11 (BD Biosciences, Cowley, Oxford, UK) with appropriate blocking steps and isotype controls and analyzed by 2-color flow cytometry (BD Biosciences, using FACSDiva software) (Fig 1, A). In a subgroup of 3 subjects with refractory asthma, the proportion of iNKT cells in the BAL and peripheral blood mononuclear cells were estimated by flow cytometry using the additional monoclonal antibodies V $\alpha$ 24 and V $\beta$ 11 (Immunotech, Luton, UK). We have found 6B11 antibodies to have high sensitivity and specificity for NKT cells based on our analysis of circulating NKT cell lines and clones.

The mean (SEM) age of the patients with asthma and the control group was 53.8 (4.7) and 45.3 (3.7) years, respectively, and the FEV<sub>1</sub> was 2.29 L (0.2) and 2.69 L (0.7), respectively. In the group with asthma, the geometric mean (logSEM) PC<sub>20</sub>FEV<sub>1</sub> methacholine (mg/mL) was 0.68 (0.3) and the sputum eosinophil percentage cell count was 4.6% (0.2). According to GINA treatment classification, the group with asthma included 2 subjects at treatment step I, 7 at step IV, and 2 at step V. Four subjects with asthma also satisfied the ATS refractory asthma criteria. Therefore, the patients with asthma were classified as mild (GINA I), moderate (GINA IV and nonrefractory), and severe refractory (GINA IV or V and refractory). The mean (SEM) percentage of BAL CD3<sup>+</sup> iNKT cells in both asthma 0.37% (0.1) and controls 0.12% (0.02) was very low, and no significant difference was found between patients with asthma and controls ( $P = .4$ ) or between mild-to-moderate and severe refractory asthma ( $P = .8$ ) (Fig 1, B). In patients where we had examined blood iNKT, no significant difference was observed between the proportion of CD3 cells that were iNKT<sup>+</sup> in PBMC and BAL cells and no difference was observed among the 3 monoclonal antibodies used (data not shown).

We report here that the proportion of iNKT cells in BAL in asthma was very low. Our findings are in contrast to the recent study by Akbari et al<sup>6</sup> that found that up to 60% of BAL CD4<sup>+</sup> cells were iNKT cells in moderate-to-severe asthma. The findings by Akbari et al<sup>6</sup> were derived by using a combination of CD1d tetramers loaded with  $\alpha$ -galactosylceramide and the monoclonal antibody 6B11 to identify BAL iNKT cells. We used the same monoclonal antibody, which makes it unlikely that the discrepant findings are simply caused by technical differences. However, the limited percentages we found are similar to the findings by investigators who have studied mild patients with asthma and children.<sup>8,9</sup> One explanation for this previous discrepancy was that the iNKT cells may be associated with more severe disease. In our study, we have carefully included subjects with mild disease and those with severe refractory asthma. Therefore, we are confident that in our study the paucity of iNKT cells does not reflect a selection bias of patients. Our inability to identify iNKT cells cannot be explained by technical problems with our choice of antibody, as we could demonstrate high sensitivity of this

antibody in cloned iNKT cells. Furthermore, our findings were not reliant on a single antibody as we confirmed our results using 3 different monoclonal antibodies. One possible criticism of our study is that we included a relatively small number of subjects. However, a *post hoc* power calculation identified that our study was powered to detect a difference greater than 0.5% in the proportion of iNKT cells in BAL between patients with asthma and controls. Therefore, we cannot exclude the possibility of a small increase in iNKT cell number in asthma, but the biological importance of such a small increase is questionable.

In conclusion, our findings do not support the view that iNKT cells are important in the pathogenesis of asthma. More studies are needed to unravel the precise role of iNKT cells in human asthma before embarking on developing treatment strategies targeted at reducing these cells.

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