

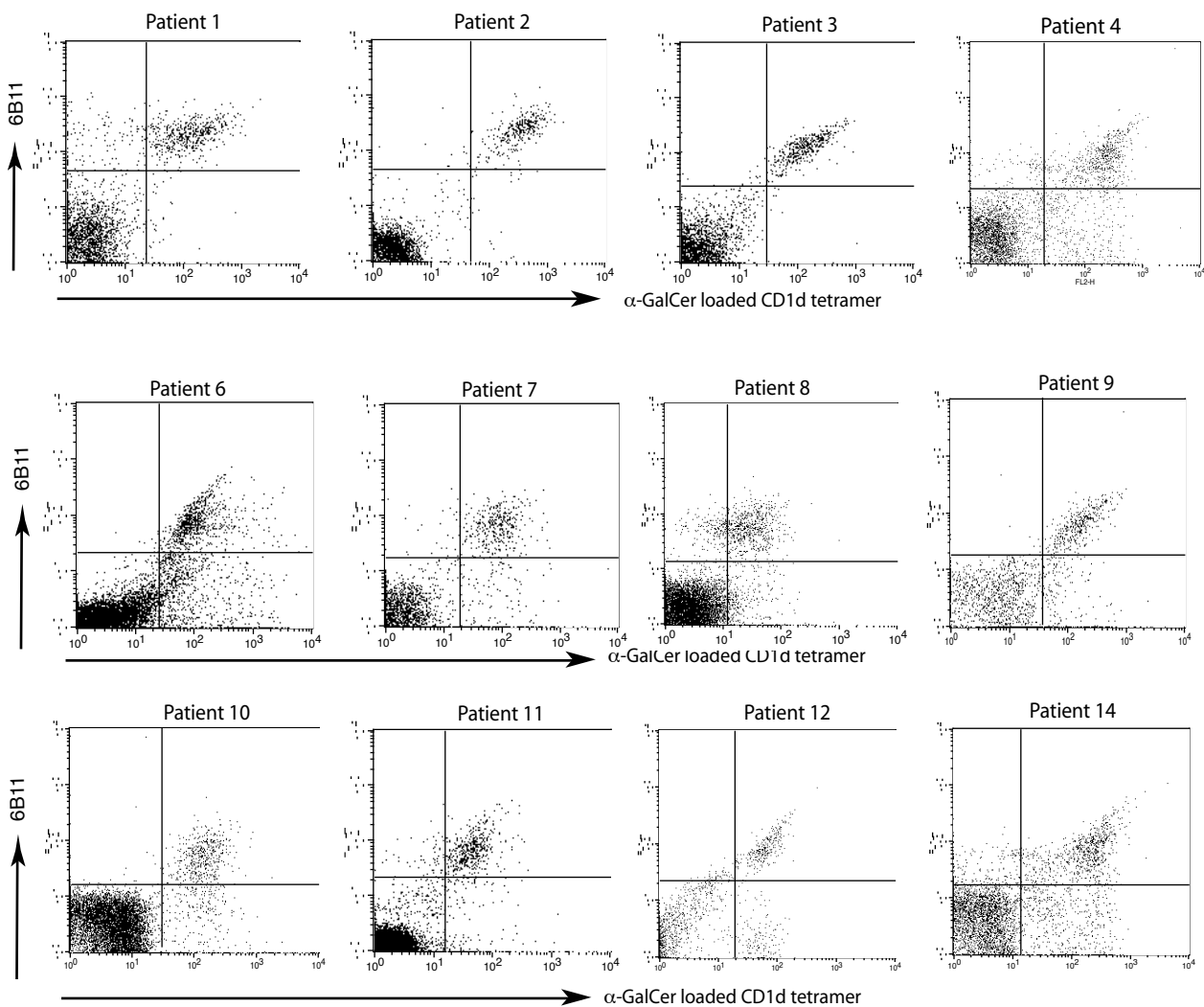
Supplementary Appendix

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

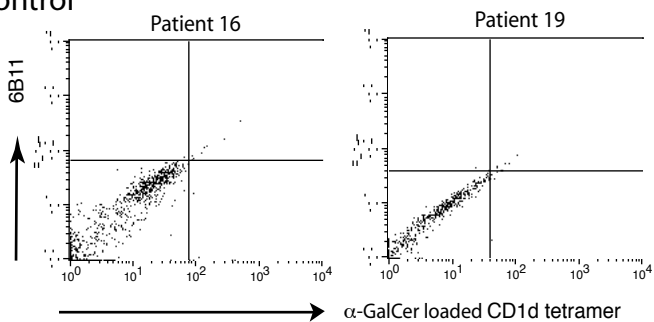
Supplement to: Akbari O, Faul JL, Hoyte EG, et al. CD4+ invariant T-cell-receptor+ natural killer T cells in bronchial asthma. *N Engl J Med* 2006;354:1117-29.

SUP. Fig 1

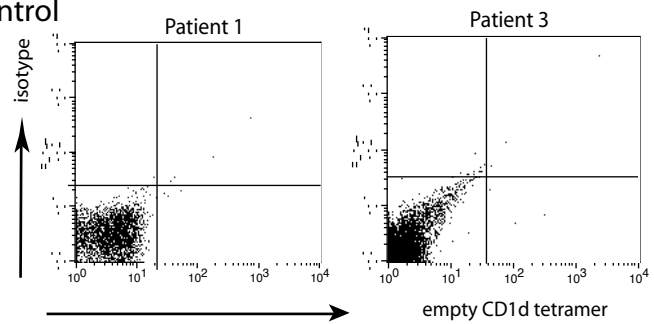
A. Asthma



B. Healthy Control



C. Isotype control



Web Appendix

Material and Methods:

Bronchial biopsy

During flexible fiberoptic bronchoscopy up to three endobronchial biopsy samples were obtained through the bronchoscope with spike and cup forceps from the second-generation right upper lobe bronchus from patients with sarcoidosis and asthma, but not healthy controls. Biopsy samples were immediately placed on sterile phosphate-buffered saline solution-moistened gauze, embedded in optimum cutting temperature medium, and snapfrozen in isopentane (precooled in liquid nitrogen). The samples were then stored in liquid nitrogen until further analysis. Cryostat sections (6 μm in thickness) of the endobronchial biopsy samples were cut at 25°C and placed onto poly-L-lysine-coated microscope slides. The slides were air dried for 60 minutes, fixed in chloroform/acetone (1:1), wrapped in cling film, and stored at 20°C until used. Each biopsy sample was sectioned within 1 month of freezing. At least 40 sections were cut and stored at minus 20°C until use. Representative sections of all samples were stained with 0.1% toluidine blue to reveal morphology and tissue integrity.

Flow Cytometric Analysis

Cells from BAL fluid were washed three times with PBS and split into fractions. Invariant NKT cells were stained with α -Galactosyl Ceramide (α -GalCer) loaded murine CD1d tetramers, which stain both human and murine NKT cells (1), as follows: Cell were incubated in 2% fetal calf serum for 20 minutes on ice, and in some experiments (patients 8, 9, 10) with purified human IgG (ChromePure Human IgG; Jackson Immunoresearch Laboratories, Inc.) 2 μg / sample, for 20 min on ice to prevent nonspecific Fc receptor binding. Cells were then incubated with 1 μg α -GalCer loaded CD1d tetramers in 100 μl for 1 h at room temperature. Other mAbs such as anti-CD4, CD8 or 6B11 (BD Bioscience) were then added at the concentration of 1 μg /100 μl for a further 30 min incubation on ice in 2% FCS. Cells were then washed with staining buffer (PBS, 0.1% BSA) and analyzed by flow cytometry using FACSsort and CELLQuest software (Becton Dickinson).

Immunohistochemical Analysis

From each bronchial biopsy, four-micrometer sections were cut, placed in acetone for one minute, and dried at room temperature for one hour. In addition to hematoxylin and eosin staining to determine tissue morphology, immunohistology was employed to identify 6B11⁺ (NKT) cells in the bronchial mucosa. For fluorescence microscopy, a FITC conjugated 6B11 mAb (green) was used. All immunohistochemistry sections were read by an investigator blinded to the diagnosis (GJB).

Molecular analysis of TCR expression

Total RNA was extracted from BAL fluid cells using Trizol (Invitrogen, California, USA). RNA (2 µg) was reverse transcribed according to standard protocols. PCR was performed semiquantitatively on the transcribed cDNA using the following conditions and primers: GeneAmp PCR System 9700 for 25, 30 or 35 cycles (60 seconds at 94°C, 60 seconds at 52°C, and 60 seconds at 72°C), Ca: GAGGGAGCACAGGCTGTCTT; TCR V α 24: GATATACAGCAACTCTGGATGCA; TCR V α 23 (an irrelevant TCR): AGTGGAAGACTTAATGCCTCGCT; TCR V β 11: TCAACAGTCTCCAGAATAAGGACG and GTGGGAGATCTCTGCTTCTG; and β -actin: ATGCCACGGCTGCTTCCAGC and CAGGAGGAGCAATGATCTTGAT.

Table 2 appendix:

Bronchoscopy was performed with an Olympus BF-XT 20 or BF-IT 30 bronchoscope, Tokyo, Japan. All subjects received an inhalation of nebulized albuterol (2.5 mg) 15 min prior to bronchoscopy. Supplementary oxygen (2 L/min) was administered via a nasal cannula and oxygenation saturation was monitored by pulse oximetry during the procedure. The BAL fluid was recovered into siliconized plastic containers, stored at 4 C and filtered through a nylon filter with a pore diameter of 100 µm (Sintab Product AB, Malmo, Sweden). Aliquots of pooled fluid were processed within 2 h of recovery for the assessment of cellular and non-cellular components. Total cell counts were performed with an automated cell counter (Technicon H1;

Technicon Instruments, Tarrytown, NY, USA). Differential cell counts were made on cytopsin preparations counting 300 cells, excluding epithelial cells.

References:

1. Brossay L, Chioda M, Burdin N, et al. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med* 1998;188:1521-8.