

Alveolar T-helper type-2 immunity in atopic asthma is associated with poor clinical control

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Abstract

Real-world evaluation studies have shown that many patients with asthma remain symptomatic despite treatment with inhaled corticosteroids (ICSs). As conventional ICSs have poor access to the peripheral airways, the aim of the present paper was to study the relationship between peripheral airway inflammation and clinical control in allergic asthma. Consequently, bronchial and transbronchial biopsies were obtained from patients with poorly controlled asthma ($n = 12$, asthma control test (ACT) score < 20), patients with well-controlled asthma ($n = 12$, ACT score ≥ 20) and healthy controls ($n = 8$). Tissue sections were immunostained to assess multiple leucocyte populations. To determine the degree of T-helper type-2 (Th2) immunity, the logarithmic value of the ratio between Th2 cells/mm² and Th1 cells/mm² was used as a surrogate score for Th2-skewed immunity. In the bronchi, the leucocyte infiltration pattern and the Th2-score were similar between patients with well-controlled asthma and those with poorly controlled asthma. In contrast, in the alveolar parenchyma, the expression of T-helper cells was significantly higher in patients with poorly controlled asthma than in patients with well-controlled asthma ($P < 0.01$). Furthermore, the alveolar Th2-score was significantly higher in patients with poorly controlled asthma (median 0.4) than in the controlled patients (median -0.10 , $P < 0.05$). In addition, in contrast with bronchial Th2-score, the alveolar Th2-score correlated significantly with ACT score ($r_s = -0.62$, $P < 0.01$) in the pooled asthma group. Collectively, our data reveal an alveolar Th2-skewed inflammation, specifically in asthmatic patients who are poorly controlled with ICSs, and suggest that pharmacological targeting of the peripheral airways may be beneficial in this large patient category.

Key words: alveolar parenchyma, asthma control asthma, peripheral inflammation, T-helper type-2 (Th2)

INTRODUCTION

Asthma is a chronic inflammatory disorder characterized by airway obstruction and hyper-responsiveness to constricting stimuli. Inhaled corticosteroids (ICSs) are the most effective anti-inflammatory medications to keep asthma under clinical control [1,2]. Yet, despite treatment with conventional ICSs, real-world evaluation studies have shown that many patients with asthma remain poorly controlled [3,4]. These patients represent a major challenge in asthma management and new therapeutic strategies are needed.

Generally, asthma is considered a disease of the large conducting airways, and much research has focused on character-

izing cellular inflammation and tissue remodelling in the bronchi of patients with asthma. This has led to the concept that asthma is driven by an aberrant T-helper type-2 (Th2) bronchial inflammation, characterized by increased numbers of Th2 cells, eosinophils and mast cells, which participate in driving the disease [5]. However, accumulating evidence suggests that, apart from the existence of disease phenotypes [6], a significant distal inflammatory component may also be present. Indeed, the few pioneering studies that have sampled tissue from the relatively inaccessible peripheral lung regions in patients with asthmatic report inflammatory cell infiltrations, both in small airways [7–10] and in the alveolar parenchyma [7–9,11,12]. Due to the invasiveness required to characterize peripheral airway inflammation,

Abbreviations: ACT, asthma control test; BDP-HFA, beclomethasone hydrofluoroalkane; BMI, body mass index; BUD-DPI, budesonide dry powder inhaler; FEV₁, forced expiratory volume in 1 s; ICS, inhaled corticosteroid; IL, interleukin; IR, immunoreactivity; NK, natural killer; NKT cell, natural killer T-cell; PD₂₀, provocative dose (metacholine) producing a fall in FEV₁ of 20% or more; Th1, T-helper type-1; Th2, T-helper type-2.

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most of these studies have involved patients with severe asthma, or asthmatic patients requiring lung resection for treatment of carcinoma. Therefore, the available data on peripheral inflammation in the less severe forms of asthma remain very scarce [13]. Of particular interest, from a therapeutic point of view, is to evaluate if the peripheral airway inflammation is present in patients with asthma that is poorly controlled with conventional ICSs, as these therapies have poor access to the lung periphery [14].

We have demonstrated that alveolar mast cells are dramatically altered in patients with poorly controlled atopic asthma compared with patients with allergic rhinitis or healthy control subjects [15,16]. Not only were the numbers of alveolar mast cells increased, but the infiltrating alveolar mast cells also displayed a marked increase in Fc ϵ RI expression and surface-bound IgE [15,16]. In the present study, we hypothesized that these distal mast cell alterations have been evoked by an underlying cellular inflammation in the alveolar parenchyma, where conventional ICSs have poor access. Consequently, with a focus on T-helper cell profiles, the aim of the present study was to, for the first time, reveal the detailed infiltration patterns of multiple infiltrating leucocyte populations in both bronchial and transbronchial biopsies in atopic asthma, and to study how these are linked to clinical control and lung function. Through a unique access to transbronchial biopsies from non-diseased control subjects, our study also allowed a direct comparison between alveolar inflammation patterns in asthma and healthy baseline conditions.

MATERIALS AND METHODS

A detailed description of the methods is available in the Supplementary Online data, which is available at <http://www.clinsci.org/cs/128/cs1280047add.htm>.

Subjects

The present study was approved by the ethics committee in Lund, Sweden (LU412-03), and all the volunteers gave written and informed consent. The asthma cohort consisted of 24 patients who were divided into two groups: 12 atopic patients with poorly controlled asthma and 12 atopic patients with well-controlled asthma. The asthma control test (ACT) was used to assess control, which is a questionnaire consisting of five questions, where the lowest and highest possible scores are 5 and 25 respectively. A cut-off score of 19 or less was used to identify patients with poorly controlled asthma and a cut-off score of 20 or more to identify patients with well-controlled asthma [4]. The ACT measures control over a period of 4 weeks and the reported scores in this study were obtained within 3 days prior to bronchoscopy. Eight healthy non-atopic subjects with negative methacholine challenge test and with no history of respiratory symptoms served as controls. All the subjects included in the present study were non-smokers and had not had an upper respiratory tract infection within 3 weeks prior to medical examination. During the year before bronchoscopy, two patients in the poorly controlled asthma group experienced one exacerbation each that required treatment with oral corticosteroids for 5 and 10 days respectively.

Bronchoscopy and collection of bronchial and transbronchial biopsies

From each patient, ten biopsies (five bronchial and five transbronchial) were taken at the Department of Respiratory Medicine and Allergology, Lund University Hospital, Lund, Sweden, during the period of May 2007–March 2008 (healthy controls) and December 2008–February 2012 (asthmatic patients). For subjects who showed positive skin-prick test (SPT) to seasonal pollen, bronchoscopy was performed outside the pollen season. Bronchial biopsies ($n = 5$) were taken from the segmental or subsegmental carina in the right lower or upper lobes and transbronchial biopsies ($n = 5$) were taken from the right lower lobe. Oxygen was given as needed during and after the procedure. Bronchoscopy was performed after local anaesthesia with a flexible bronchoscope (IT160; Olympus) and transbronchial biopsies were taken with biopsy forceps (FB211D; Olympus) under fluoroscopic guidance in the peripheral right lower lobe at a distance > 2 cm from the chest wall. Fluoroscopy of the right lung was performed immediately and 2 h after the procedure to rule out significant bleeding or pneumothorax. Sections (3 μ m thick) from each tissue block were stained with Mayer's Haematoxylin with the sole purpose to, in an unbiased manner, select four biopsies per patient (two bronchial and two transbronchial) with well-preserved morphology for serial sectioning and subsequent immunohistochemistry. In bronchial biopsies, well-preserved morphology was defined as the presence of both intact epithelium and lamina propria tissue, and lack of any significant mechanic crush artifacts. In the transbronchial biopsies, well-preserved morphology was defined as the presence of intact alveolar parenchyma. From the healthy controls and patients with poorly controlled asthma, two bronchial and two transbronchial biopsies could be obtained from each patient. From the patients with well-controlled asthma, two bronchial biopsies were selected from 11 out of the 12 patients and two transbronchial biopsies were selected from nine out of the 12 patients. From each of the three remaining patients, at least one bronchial and one transbronchial biopsy of high quality were analysed.

Immunohistochemistry

Immunohistochemistry was used to assess all major populations of infiltrating leucocytes. All antibodies used in the present study (Supplementary Table S1 at <http://www.clinsci.org/cs/128/cs1280047add.htm>) have been routinely used for staining of human paraffin-embedded tissue sections in research and clinical diagnosis, or validated thoroughly in our laboratory. Separate tests ruled out that the age differences between paraffin-tissue blocks had any influence of the resulting staining quality. All antibodies are commercially available, except antibodies to basophilic granules (clone BB1; Immunopharmacology Group, Southampton General Hospital, Southampton, U.K.), which recognize a unique granule constituent of basophils [18] and antibodies to eosinophil cationic protein (clone EG2; Pharmacia-Upjohn Diagnostics) [19]. Both of these antibodies have been used previously on bronchial biopsies [20–22]. To detect T-helper type-1 (Th1) and Th2 cells, CD4⁺ T-helper cells were double-stained with lineage-specific transcription factors T-Bet (to identify Th1 cells) and GATA-3 (to detect Th2 cells)

Table 1 Clinical characteristics of asthmatic patients and healthy controls

NS, not significant.

Parameter	Healthy controls	Well-controlled asthma	Poorly controlled asthma	P*
Sample size	8	12	12	–
Gender (male:female) (n)	3:5	8:4	6:6	NS
Age (years)	23 (21–39)	27 (20–40)	49 (30–59)	<0.0001
BMI (kg/m ²)	23.01 (19.38–27.10)	24.33 (20.55–29.40)	22.50 (19.59–24.97)	<0.05
FEV ₁ % pred.	98.05 (72.10–116.40)	85.95 (72.50–108.80)	81.25 (63.30–108.00)	NS
FEV ₁ (litres)	3.70 (2.38–5.38)	3.80 (2.35–5.15)	2.87 (1.84–4.64)	<0.05
PD ₂₀ (μg)	2000†	773.0 (0.1–2000)	190.8 (0.1–1620)	NS
Atopy (yes/no) (n)	0/8	12/0	12/0	NS
ACT score	–	21 (20–24)	13.5 (5–19)	–
ICS (BUD-DPI; μg/day)	–	720 (320–960)	720 (160–1120)	NS
Never-smokers/ex-smokers (n)	8/0	11/1	11/1	NS

*Well-controlled asthma compared with poorly controlled asthma.

†All patients had an unspecified PD₂₀ value greater than 2000 μg.

[23]. The immunostaining was performed identically and simultaneously for all patient groups in an automated immunohistochemistry robot operating at room temperature (Autostainer LV-1; Dako).

Tissue analysis

Stained sections were digitally scanned in an automated digital slide-scanning robot (Scanscope CS, Aperio Technologies) operating with a ×20 microscope lens and images were analysed in ImageScope (v. 10.0.36.1805; Aperio Technologies). For single-stained sections, a positive-staining-recognizing algorithm was set, based on chromogen colour and staining intensity for each leucocyte marker. The image analysis software automatically calculated the number of positive pixels and negative pixels, automatically excluding non-tissue areas such as spaces of air. The expression of leucocytes was determined by dividing the number of positive pixels with the total number of pixels, giving a percentage of immunoreactivity (IR). In the bronchial biopsies, IR was determined in the whole tissue section, subepithelium and intact epithelium cells. In the transbronchial biopsies, IR was determined in the alveolar parenchyma. Eosinophils, basophils, Th1 cells and Th2 cells were counted manually in a blinded approach and then normalized to the tissue area determined in the image analysis software. Importantly, blood, glands and cartilage were excluded from the analysis.

Th2-score calculation

The logarithmic value of the ratio between the number of Th2 and Th1 cells/mm², in tissue-sections adjacent to each other during serial sectioning, was used as a surrogate score for Th2-immunity. In patients where two bronchial or two transbronchial biopsies were analysed, the arithmetic mean of the two logarithmic values was used as the final score. In cases where one of the biopsies contained zero Th1 or Th2 cells/mm², a Th2-score of 1 (in case of 0 Th1 cells/mm²) or –1 (in case of 0 Th2 cells/mm²) was given.

Statistical Analysis

Data were analysed in GraphPad Prism v.6 (GraphPad Software) and values are given as medians (range), unless otherwise stated.

To detect significant differences between the two groups, the non-parametric two-tailed Mann-Whitney *U* test was performed. The Spearman rank (*r_s*) correlation test (two-tailed) was used to detect significant correlations between Th2-scores and clinical parameters. Statistical significance was set at *P* < 0.05.

RESULTS

Subject characteristics

The clinical characteristics of the study groups are presented in Table 1. All asthmatic patients were atopic and all, except two, were airway hyper-responsive. Compared with the well-controlled asthma group, the poorly controlled asthma group was older (median 49 as compared with 27 years) and had lower body mass index (BMI; median 22.5 as compared with 24.3 kg/m²). Pre-bronchodilator forced expiratory volume in 1 s (FEV₁) was lower in the poorly controlled asthma group, but the predicted value of pre-bronchodilator FEV₁ was similar between the poorly controlled asthma group (median 81.3%) and the well-controlled asthma group (median 86.0%). All asthmatic patients were treated with a budesonide dry-powder inhaler (BUD-DPI), except one patient with well-controlled asthma who was treated with beclomethasone hydrofluoroalkane (BDP-HFA). The equivalent dose of BUD-DPI did not differ between the well-controlled asthma (median 720 μg/day) and poorly controlled asthma (median 720 μg/day). The healthy control subjects were all non-atopic and lacked airway hyper-responsiveness [i.e. provocative dose (metacholine) producing a fall in FEV₁ of 20% or more (PD₂₀) > 2000 μg]. All subjects in the present study were non-smokers and only two individuals had a history of smoking (ex-smokers of at least 1 year prior to biopsy).

Leucocyte infiltration patterns in the large airways

The expression of infiltrating leucocytes in the whole bronchial tissue compartment in well-controlled and poorly controlled asthma is presented in Figure 1 and examples of images of the different immunostained leucocyte populations are shown in Figure 2. Except for basophils, which tended to be

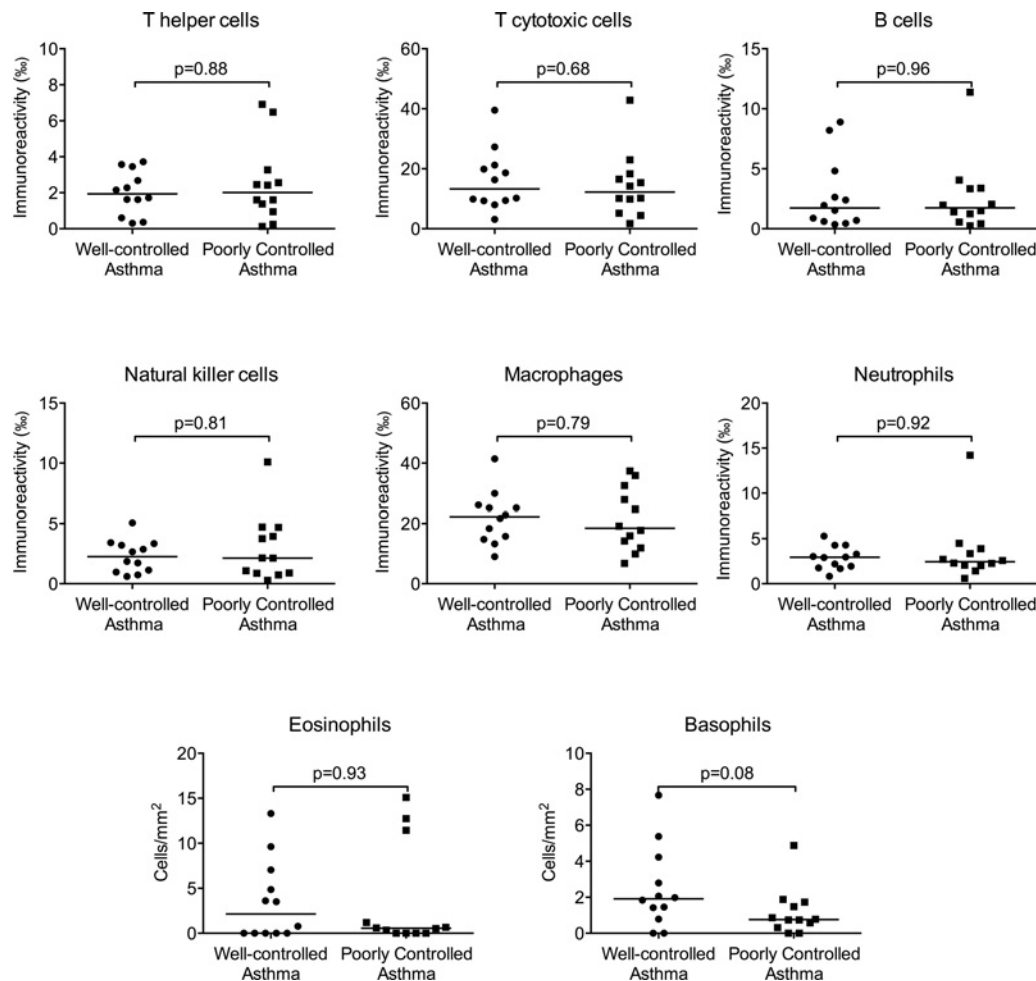


Figure 1 Scattergrams showing leucocyte infiltration in the bronchial airways in patients with well-controlled and poorly controlled asthma

Each dot represents individual mean values and horizontal bars represent the median value for each patient group.

higher in patients with well-controlled asthma, the expression CD4⁺ T-helper cells, CD8⁺ T-cytotoxic cells, B-cells, natural killer (NK) cells, macrophages, neutrophils and eosinophils was similar between well-controlled and poorly controlled asthma groups ($P \geq 0.7$ for all outcomes) (Figure 1). In the subepithelial compartment and intact epithelium, no significant differences were observed between the two asthma groups (Supplementary Table S2 at <http://www.clinsci.org/cs/128/cs1280047add.htm>). In the comparison between healthy controls and the pooled asthma group, the asthma group had increased expression of CD4⁺ T-helper cells ($P < 0.001$), NK cells ($P < 0.05$) and basophils ($P < 0.01$) and lower expression of neutrophils ($P < 0.01$) in the whole bronchial tissue compartment (Supplementary Table S3 at <http://www.clinsci.org/cs/128/cs1280047add.htm>).

Leucocyte infiltration pattern in the alveolar parenchyma

The alveolar expression of leucocytes in patients with well-controlled and poorly controlled asthma is presented in Fig-

ure 3. The expression of CD4⁺ T-helper cells was significantly increased in poorly controlled asthma compared with well-controlled asthma ($P < 0.01$). Basophils were few in numbers but significantly higher in patients with well-controlled asthma ($P < 0.05$). In comparison with healthy controls, the pooled asthma group was associated with increased expression of CD4⁺ T-helper cells ($P < 0.01$) and NK cells ($P < 0.05$) (Supplementary Table S4 at <http://www.clinsci.org/cs/128/cs1280047add.htm>). Borderline significant differences were observed in the numbers of eosinophils ($P = 0.05$) and basophils ($P = 0.05$), which tended to be higher in the asthmatic patients (Supplementary Table S4).

Bronchial and alveolar Th2-scores and correlations with clinical parameters

In the bronchial airways, the Th2-score was similar between patient with well-controlled asthma (median 0.37) and poorly controlled asthma (median 0.22; $P = 0.3$) (Figure 4A). In contrast, in the alveolar region, the Th2-score was significantly

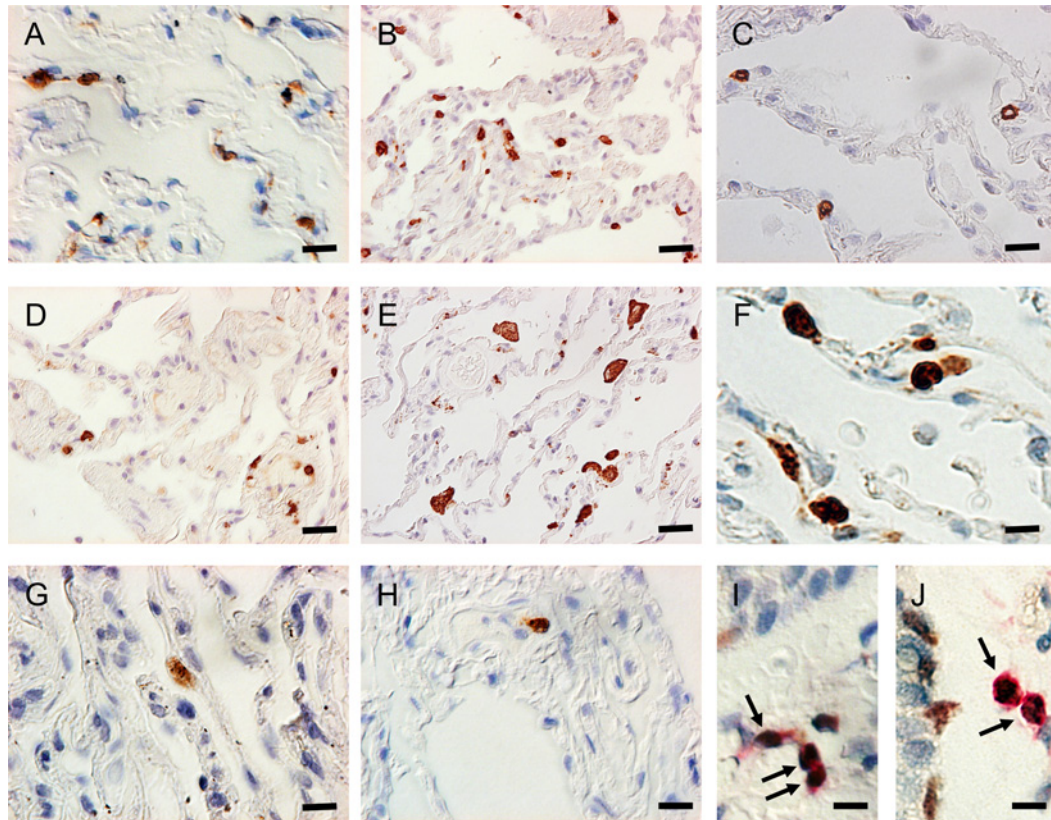


Figure 2 Bright field micrographs exemplifying the immunohistochemical staining of (A) CD4⁺ T-helper cells, (B) CD8⁺ T-cytotoxic cells, (C) CD20⁺ B-cells, (D) CD57⁺ NK cells, (E) CD68⁺ macrophages, (F) MPO⁺ neutrophils, (G) EG2⁺ eosinophils, (H) BB1⁺ basophils, (I) GATA3⁺ and CD4⁺ Th2 cells (arrows) and (J) T-Bet⁺ and CD4⁺ Th1 cells (arrows)

In all cases, the primary IR is visualized with DAB (3,3'-diaminobenzidine) chromogen (brown) and Htx staining was used as counter stain. For the double staining in (I) and (J), the second marker (i.e. CD4) is visualized with Fast Red Chromogen. Scale bars: (A), (C) and (H) = 25 μ m; (B), (D) and (E) = 30 μ m; (F) = 10 μ m; (G) and (I) = 20 μ m; and (J) = 15 μ m.

higher in poorly controlled asthma (median 0.4) compared with well-controlled asthma (median -0.10 ; $P < 0.05$) (Figure 4B). In the pooled group of asthmatic patients, alveolar Th2-score correlated significantly with ACT score ($r_s = -0.62$, $P < 0.01$) whereas bronchial Th2-score did not ($r_s = 0.03$, $P = 0.9$) (Figures 4C and 4D). Alveolar Th2-score also correlated with FEV₁ ($r_s = -0.63$; $P \leq 0.001$) and predicted FEV₁% ($r_s = -0.43$; $P < 0.05$), whereas bronchial Th2-score did not (Supplementary Figure S1 at <http://www.clinsci.org/cs/128/cs1280047add.htm>). Borderline significant correlations were found between both alveolar and bronchial Th2-scores to PD₂₀ ($r_s = -0.42$, $P = 0.05$; and $r_s = -0.39$, $P = 0.07$ respectively) in the pooled group of asthmatic patients who were airway hyper-responsive ($n = 22$) (Supplementary Figure S1). In comparison with healthy controls, the bronchial Th2-score in the pooled group of asthmatic patients (median 0.28) was similar to the controls (median = 0.49, $P = 0.13$) whereas the alveolar Th2-score in the pooled asthmatic patients (median 0.09) was higher compared with controls (median -0.15 , $P < 0.05$) (Supplementary Figure S2 at <http://www.clinsci.org/cs/128/cs1280047add.htm>).

Th2-score in the individual biopsies

The bronchial and alveolar Th2-scores in the individual biopsies from healthy controls, patients with well-controlled asthma and patients with poorly controlled asthma are presented in Figures 5(A) and 5(B) respectively.

DISCUSSION

To the best of our knowledge, this is the first study that explores the pattern of infiltrating leucocytes and Th2 immunity in both bronchial and transbronchial biopsies from asthmatic patients with variable degrees of clinical control and healthy control subjects. Although our data show that the infiltration of leucocytes is virtually the same between patients with well-controlled asthma and those with poorly controlled asthma in the bronchial airways, the alveolar parenchyma in poorly controlled asthma is associated with increased expression of CD4⁺ T-helper cells and a Th2-biased immune profile. These data suggest that ICSs control bronchial inflammation in both groups of asthmatic patients

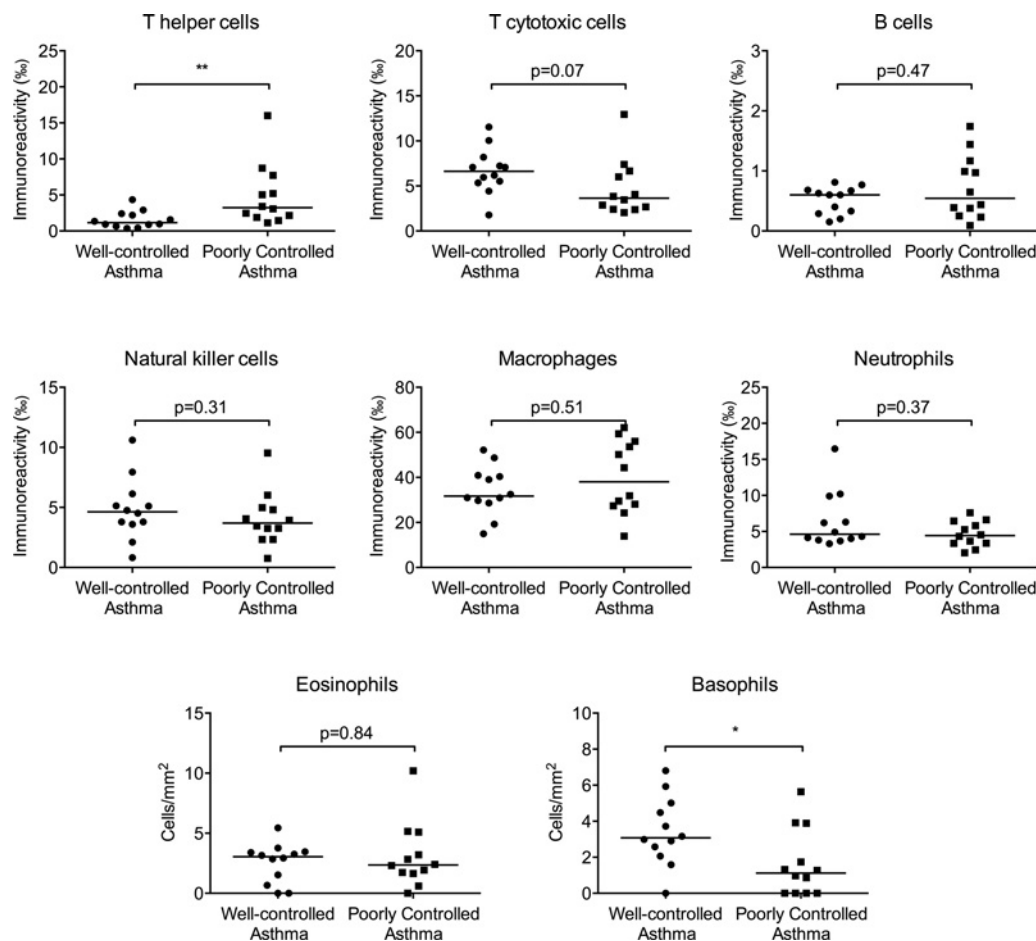


Figure 3 Scattergrams showing leucocyte infiltration in the alveolar parenchyma in patients with well-controlled and poorly controlled asthma

Each dot represents individual mean values and horizontal bars represent the median value for each patient group.

* $P < 0.05$, ** $P < 0.01$.

similarly and that treatment strategies aiming to control inflammation in the most peripheral parts of the lungs may be needed to achieve improved disease control in patients who are poorly controlled.

Th2 cells are considered to be key players in the orchestration of allergic-asthma through the secretion of a distinct repertoire of cytokines, including interleukin (IL)-4, IL-5, IL-9 and IL-13 [24]. In the present study, we used the ratio of Th2 and Th1 cells to determine Th2-skewed immunity. Previous studies have used similar strategies to determine the degree of Th2 immunity in asthmatic patients [25,26]. However, to our knowledge, this is the first study that investigates the ratio of Th2 and Th1 cells by immunohistochemistry in biopsies from asthmatic patients. Our main finding of Th2-skewed immunity in the alveolar parenchyma in poorly controlled asthma raises the question whether allergic reactions may take place in this part of the lung. Although common asthma allergens, such as house dust mite and pollen grains, are generally considered too large to reach the distal airways; it has been shown that other smaller breathable particles, such as cat allergen, are more easily deposited in the peripheral airways [27]. Moreover, air pollutants such

as diesel exhaust may facilitate the deposition of allergen fragments into the alveolar region [28–30]. Whether such peripheral allergen deposition contributes to heightened alveolar Th2 in the present study remains to be explored. In any case, if deposited in alveolar regions, common asthma allergens should be expected to initiate an adaptive immune response and mast cell activation [15]. In regard of potential alveolar mast cell activation, we discovered a significant expansion of FcεRI-expressing mast cells with elevated surface-bound IgE in the alveolar parenchyma in uncontrolled asthmatic patients [15]. Notably, alveolar mast cells in healthy human lungs virtually lack expression of FcεRI and surface-bound IgE and should consequently be regarded as less capable to mount a classical IgE-mediated induction of an allergic response [15,31]. Taking the data together, although the concept of alveolar inflammation in asthma is intriguing, it remains to be determined to what extent allergic immune triggers directly contribute to the altered alveolar immune cell-profile in asthma. In this important search, attention must be paid also to the possibility of passive diffusion of Th2-promoting mediators from neighbouring conducting, or non-allergic Th2 triggers.

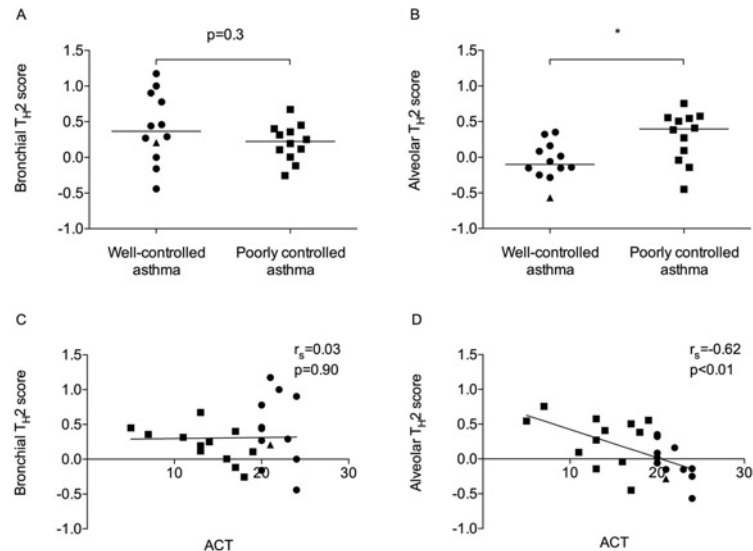


Figure 4 Scattergrams showing Th2-scores (A and B) and correlation between Th2-score and ACT score (C and D) in well-controlled and poorly controlled asthma in bronchial airways and alveolar parenchyma respectively. Each dot represents individual mean values and horizontal bars represent the median value for each patient group. The triangle represents the patient with well-controlled asthma who was treated with BDP-HFA. * $P < 0.05$.

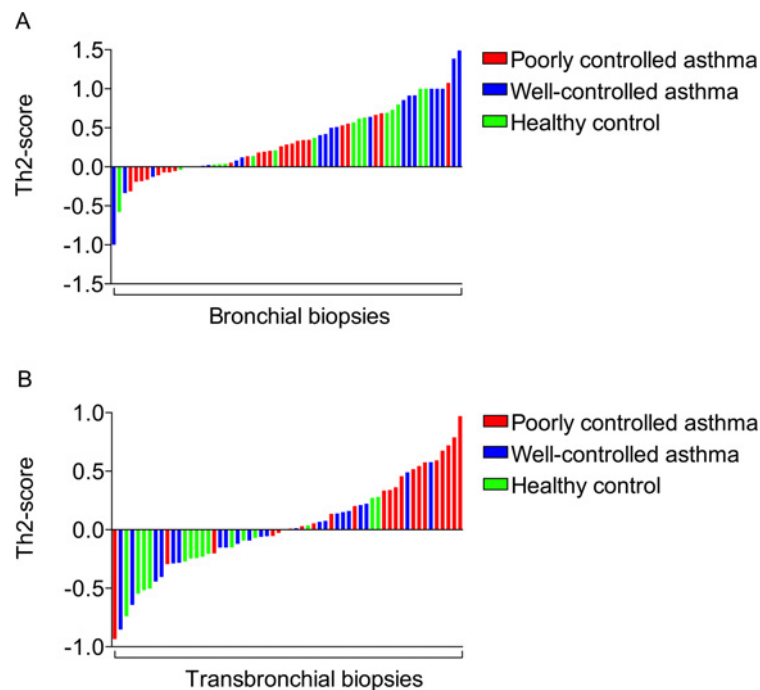


Figure 5 Th2-scores in the individual biopsies from healthy controls and patients with well-controlled and poorly controlled asthma in the bronchial (A) and transbronchial (B) biopsies respectively.

Despite the increased alveolar Th2-score in poorly controlled asthma, alveolar eosinophils were not increased compared with well-controlled asthma. This observation, which may seem surprising in light of the proposed link between Th2 immunity and eosinophilia, could be explained by differences in cell dynamics. Th2 cells are long-lived and reside in the tissue, whereas eosino-

phils are short-lived and exhibit a different and more fluctuating tissue dynamic [32,33]. This is also elegantly shown in patients with nocturnal asthma where, particularly, the number of alveolar eosinophils fluctuated during the day [11]. Due to differences in cell dynamics between Th2 cells and eosinophils, a larger sample size than used in present study may be needed to demonstrate a

connection between Th2 immunity and eosinophils in the alveolar parenchyma.

Besides initiating a local inflammatory response, activated leucocytes in the alveolar parenchyma could potentially give rise to a 'leakage' of pro-inflammatory cytokines into the blood stream. Considering the large surface area of the alveoli, this could lead to a certain degree of systemic inflammation. Although the role of systemic inflammation in asthma is largely unknown, it has been shown that patients with severe asthma have increased serum levels of pro-inflammatory cytokines compared with patients with mild-to-moderate asthma [34]. Interestingly, elevated levels of pro-inflammatory cytokines in the blood stream can affect the quality of life, as well as other factors such as sleep [35].

One potential drawback with the present study is the small sample size of healthy controls and asthmatic patients, which makes generalizability of the findings in the present study an issue. However, due to the invasiveness required to sample trans-bronchial biopsies and the unique and exploratory nature of the present study, we believe that the number of patients is appropriate in the present study, which clearly provides a rationale for follow-up studies to investigate more on the importance of alveolar Th2 inflammation in asthma. Furthermore, as this is a cross-sectional study, it remains to be determined if alveolar Th2 inflammation changes over time with more effective corticosteroid treatment and how this is associated with improved disease control.

Another issue that should be addressed is that, although the asthma groups were well matched in most clinical characteristics, the patients with poorly controlled asthma were older than patients with well-controlled asthma. Old age is a potential confounding factor when evaluating asthma control as it has been shown that short- and long-term control is worse in patients 65 years or older compared with patients between 18 and 64 years of age [36]. In the present study, none of the patients with poorly controlled asthma were 65 years or older. It should also be mentioned that in a large European multi-centre study, where the mean age was 39.9 years, no association between age and ACT score was found [37]. The mean age of the pooled-group of asthmatic patients in our study was 37.7 years (results not shown). For these reasons, we believe that variation in ACT score is not reflected by variation in age in the present study. Another concern regarding the difference in age in the present study that needs to be addressed is the phenomenon of 'immunosenescence', which is a term used to describe changes in inflammatory status that are associated with aging [38]. Although age-related changes of the immune function in human asthma are yet to be studied in detail, there are studies in animal models and humans suggesting that age can affect both innate and adaptive immune responses [39]. Features of immunosenescence that may be important in asthma have been reviewed [39] and are to include changes in neutrophils, NK cells, natural killer T-cells (NKT cells), monocytes/macrophages, eosinophils, dendritic cells, T-cells and B-cells. In terms of cell numbers, NK cells and memory CD8 T-cells are suggested to increase with age whereas NKT cells are suggested to decrease [40–42]. In present study, we found no significant difference in the expression of NK cells or CD8⁺ cells in the alveolar parenchyma (which is expected to be relatively unaffected by the ICS treatment) between the two asthma groups, which strongly

argue against a significant role of immunosenescence in this tissue compartment in the patients with poorly controlled asthma. In terms of cytokine profiles in CD4⁺ T-cells, it has been shown in healthy subjects in three different age groups (21–31 years, 80–81 years and 100–103 years) that the ratio between type-2 cytokines and type-1 cytokines is unaffected by age [43]. As Th2 cells are the major source of type-2 cytokines and Th1 cells are the major source for type-1 cytokines, the present study suggests that there is no inherited bias of CD4⁺ cells to differentiate into Th2 cells with increasing age. Thus, it seems more likely that the increased Th2/Th1 profile among the CD4⁺ cells in the alveolar parenchyma in the poorly controlled asthma group is attributed by the disease of atopic asthma rather than by older age.

In summary, the present study shows that alveolar Th2 immunity is associated with poor clinical control in atopic asthma. Although the delicate and invasive nature of this type of study limits the number of patients who can be included, we were able to obtain statistically secured and clear results for all main parameters. Although the mechanisms by which alveolar Th2 immunity can affect clinical manifestations of atopic asthma remains to be determined, our data indicate that pharmacological targeting of the peripheral airways may benefit patients who remain poorly controlled with standard ICS treatment.

CLINICAL PERSPECTIVES

- Asthma is still mainly regarded and pharmacologically treated as an inflammatory disease of the bronchial airways.
- The present study provides new immunological data to suggest that this view is too simplistic and that an inflammatory component in the distal lung should also be considered. Specifically, through a unique sampling of alveolar tissue, we demonstrate that poor clinical control in atopic asthma is linked with a Th2-skewed alveolar tissue inflammation.
- This finding is suggested to have important bearings to the development of new treatment strategies as conventional ICSs mainly target the bronchial airways and have poor access to the peripheral airways.

AUTHOR CONTRIBUTION

Anders Bergqvist: design of the study, performance and interpretation of experiments, writing the manuscript. Jonas Erjefält: design of the study, interpretation of experiments, writing the manuscript. Leif Bjermer: collection of biopsies, and reviewing the manuscript for important intellectual content. Cecilia Andersson, Michiko Mori and Andrew Walls: reviewing the manuscript for important intellectual content.

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SUPPLEMENTARY ONLINE DATA

Alveolar T-helper type-2 immunity in atopic asthma is associated with poor clinical control

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MATERIALS AND METHODS

Allergy screening

A standardized skin-prick test (ALK-Abelló) was used on all subjects to screen for sensitization of ten inhalant allergens (birch, timothy, mugwort, cat, dog, horse, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Aspergillus fumigatus* and *Cladosporium herbarum*). Atopy was defined as a positive response (weal reaction larger or equal to histamine-positive control) to one or several allergens.

Spirometry and methacholine inhalation challenge

Lung function was measured with a MasterScope spirometer (version 4.5; Erich Jaeger) and reference values were obtained from Crapo et al. [1]. Two FEV₁ values were recorded with less than 4% variation and the better value was recorded as the baseline. Bronchial hyper-responsiveness was measured with a methacholine inhalation challenge test performed with a tidal volume-triggered instrument (Aerosol Provocation System; Erich Jaeger). The Aerosol Provocation System delivered a cumulative dose of 2000 µg of methacholine in five increments (50, 150, 300, 600 and 900 µg) after an initial dose of 0.9% NaCl. A positive test was defined as the cumulative dose that caused the FEV₁ to decline by 20% or more from the baseline. When FEV₁ declined less than 80% of the baseline value or when the total amount of methacholine (2000 µg) was delivered, 400 µg of salbutamol was given to the subject. After 10 min, a new flow-volume spirometry was carried out to ensure proper patient recovery.

Tissue processing

From each subject, five bronchial and five transbronchial biopsies were collected and were, immediately after excision, placed in 4% buffered formaldehyde (fixation time, 1.5–2 h at room temperature and 15–19 h at 4°C), dehydrated and embedded in

paraffin. Sections (3 µm thick) from each tissue block were stained with Mayer's Haematoxylin with the sole purpose to, in an unbiased manner, select biopsies with well-preserved morphology for serial sectioning and subsequent immunohistochemistry. In bronchial biopsies, well-preserved morphology was defined as presence of both intact epithelium and lamina propria tissue. In the transbronchial biopsies, well-preserved morphology was defined as the presence of intact alveolar parenchyma. From the healthy controls and patients with poorly controlled asthma, two bronchial and two transbronchial biopsies were selected from each patient. From the patients with well-controlled asthma, two bronchial biopsies were selected from 11 out of the 12 patients and two transbronchial biopsies were selected from nine out of the 12 patients. From each of the few remaining patient, one bronchial biopsy and one transbronchial high-quality biopsy were analysed.

Single-staining immunohistochemistry

Sections were incubated at 60°C for 20 min and then pre-treated with heat-induced antigen retrieval in DIVA decloaker (Biocare Medical), pH 6, in a pressure cooker (2100 Retriever; Prestige Medical) except sections subjected for CD57 staining, which were deparaffinized in a series of xylene and alcohol baths. All single staining experiments were performed using the EnVision™ Detection System Peroxidase/DAB, Rabbit/Mouse (K5007; Dako) according to the manufacturer's instructions. In brief, sections were blocked with 0.3% hydrogen peroxide (Merck) for 10 min, incubated with primary antibody (1 h), incubated with a dextran polymer reagent containing peroxidase molecules and secondary anti-mouse and anti-rabbit antibodies (30 min) and then finally incubated with non-permeant chromogen DAB (8 min). Background staining was visualized with haematoxylin and sections were taken through graded alcohol baths (3 × 5 min) and xylene baths (3 × 5 min) before being mounted with Pertex mounting medium (Histolab Products).

Double-staining immunohistochemistry

Sections were incubated at 60 °C for 20 min and then pretreated (deparaffinized, rehydrated and antigen retrieved) in pH 9.0 using a PT Link (Dako). Sections were then stained with either T-Bet or GATA-3 antibodies using the EnVision™ Detection System Peroxidase/DAB, Rabbit/Mouse (K5007; Dako) as described above. Next, a double-staining blocking solution (Biocare Medical) was added (5 min) to denature residual secondary antibodies on the dextran polymer to avoid cross-reactivity. Sections were also treated with 20% horse serum for 20 min (Vector Laboratories) and avidin/biotin blocking reagents (Dako) for 10 min respectively, to avoid cross-reactivity. The sections were

then incubated with antibodies towards CD4 for 1 h and then with biotinylated horse anti-mouse secondary antibodies (Vector Laboratories) at a 1:200 dilution for 1 h. After this, sections were treated with streptavidin-alkaline phosphatase solution (AbD Serotec) at 1:100 dilution for 40 min and finally with permanent Red Chromogen (Dako) at 1:100 dilution for 10 min. Background staining was visualized with haematoxylin and sections were dried at 60 °C before taken through xylene baths (3 × 5 min) and finally mounted with Pertex mounting medium (Histolab Products). Staining with matched isotype control antibodies for the T-Bet and GATA-3 antibodies resulted in no immunostaining.

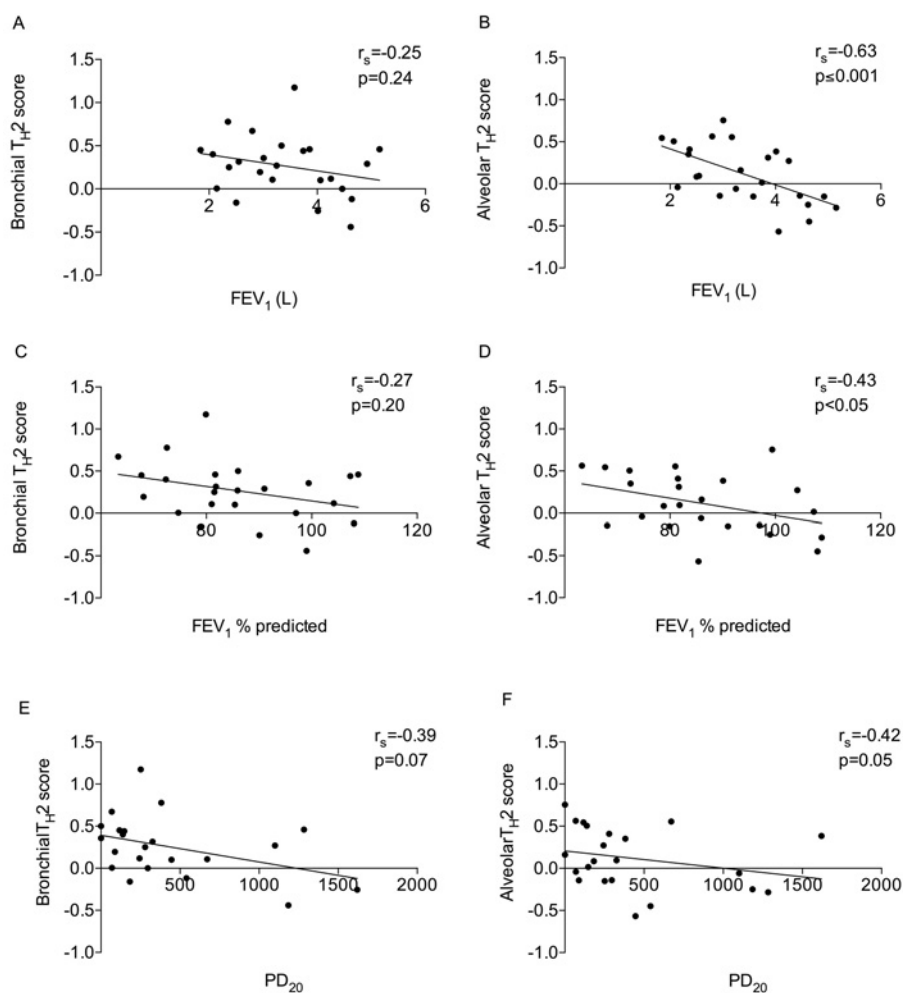


Figure S1 Correlations between Th2-score and FEV_1 or PD_{20}

(A and B) Correlation between Th2-score and FEV_1 (litres) in the pooled group of asthmatic patients in the bronchial airways and alveolar parenchyma respectively. (C and D) Correlation between Th2-score and FEV_1 % predicted in the pooled group of patients with asthma in the bronchial airways and alveolar parenchyma respectively. (E and F) Correlation between Th2 score and PD_{20} in the pooled group of asthmatic patients that were airway hyper-responsive (i.e. $PD_{20} < 2000 \mu g$) in the bronchial airways and alveolar parenchyma respectively. Each dot represents individual mean values.

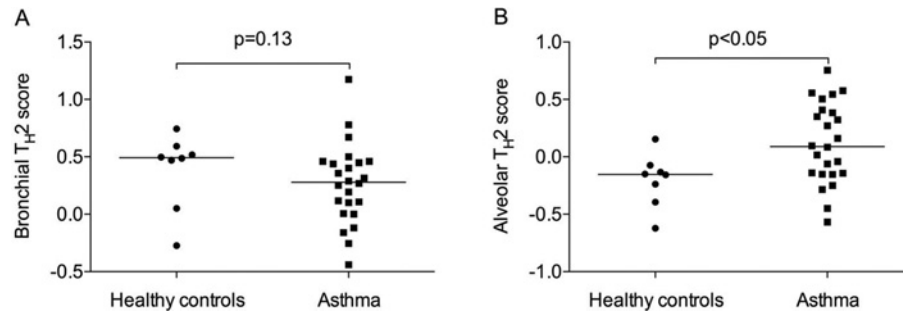


Figure S2 Bronchial and alveolar Th2-scores in healthy controls and the pooled group of patients with asthma
Each dot represents individual mean values and the horizontal bar represents the median value for each respective group.

Table S1 Primary antibodies used in immunohistochemistry

Antibody	Marker for	Species	Dilution	Clone/catalogue no.	Manufacturer
CD4	T-helper cells	Mouse IgG1	1:100	1F6	Novocastra
CD8	T-cytotoxic cells	Mouse IgG1 kappa	1:640	C8/144b	Dako
CD20	B-cells	Mouse IgG2a kappa	1:2000	L26	Dako
CD57	NK cells	Mouse IgM	1:200	NK-1	Novocastra
CD68	Macrophages	Mouse IgG3 kappa	1:1200	PG-M1	Dako
MPO	Neutrophils	Rabbit polyclonal	1:20000	A0398	Dako
EG2	Eosinophils	Mouse IgG1	1:1000	EG-2	Pharmacia-Upjohn Diagnostic
BB1	Basophils	Mouse IgG2a	1:50	BB1	Andrew Walls Laboratory
T-Bet	Th1-differentiated T-helper cells	Rabbit polyclonal	1:4000	H-210	Santa Cruz Biotechnology
GATA-3	Th2-differentiated T-helper cells	Mouse IgG1 kappa	1:5000	L50-823	BD Pharmingen
Isotype control	Non-human antigen	Mouse IgG1 kappa	1:1000	X0931	Dako
Isotype control	Non-human antigen	Rabbit IgG	1:10	NP-0001	Epitomics

Table S2 Leucocyte infiltration in the subepithelium and intact epithelium in well-controlled and poorly controlled asthma
Values are given as medians (range) unless otherwise stated.

Leucocyte*	Subepithelium			Epithelium		
	Well-controlled asthma (n = 12)	Poorly controlled asthma (n = 12)	P	Well-controlled asthma (n = 12)	Poorly controlled asthma (n = 12)	P
T-helper cells	2.4 (0.3–4.5)	2.3 (0.0–8.2)	1.0	0.2 (0.0–3.8)	0.3 (0.0–2.3)	0.64
T-cytotoxic cells	13.8 (3.0–41.2)	11.2 (1.0–49.0)	0.47	17.2 (7.3–45.0)	19.6 (4.8–41.8)	0.96
B-cells	2.2 (0.5–10.8)	2.0 (0.3–13.0)	0.83	0.0 (0.0–8.2)	0.1 (0.0–3.4)	0.26
NK cells	1.4 (0.6–3.3)	1.8 (0.3–10.6)	0.43	1.4 (0.1–11.9)	1.4 (0.0–4.8)	0.51
Macrophages	24.7 (9.3–47.2)	20.6 (7.2–41.7)	0.74	9.8 (1.2–33.6)	11.4 (4.9–35.8)	0.51
Neutrophils	3.0 (0.9–5.7)	2.6 (0.7–14.5)	0.70	1.1 (0.0–8.1)	2.2 (0.1–12.0)	0.12

*Given as % immunoreactivity.

Table S3 Leucocyte infiltration in the bronchial airways in healthy controls and asthmatic patients

Values are given as medians (range) unless otherwise stated.

Leucocyte	Whole biopsy			Subepithelium			Epithelium		
	Controls (n = 8)	Asthma (n = 24)	P	Controls (n = 8)	Asthma (n = 24)	P	Controls (n = 8)	Asthma (n = 24)	P
T-helper cells (% IR)	0.3 (0.0–1.7)	1.9 (0.1–6.9)	< 0.001	0.3 (0.0–7.1)	2.4 (0.0–8.2)	< 0.05	0.0 (0.0–1.4)	0.3 (0.0–3.8)	< 0.01
T-cytotoxic cells (% IR)	21.2 (6.4–54.7)	12.2 (1.7–42.9)	0.12	23.7 (6.0–64.7)	12.0 (1.0–49.0)	0.07	20.1 (2.8–37.6)	19.0 (4.8–45.0)	0.59
B-cells (% IR)	4.0 (0.3–11.6)	1.7 (0.2–11.4)	0.15	3.5 (0.4–15.1)	2.2 (0.3–13.0)	0.24	0.3 (0.0–2.5)	0.1 (0.0–8.2)	0.72
NK cells (% IR)	1.1 (0.2–1.8)	2.1 (0.3–10.1)	< 0.05	0.9 (0.2–1.8)	1.5 (0.3–10.6)	0.05	1.1 (0.0–1.8)	1.4 (0.0–11.9)	0.40
Macrophages (% IR)	18.3 (4.6–44.8)	20.4 (6.8–41.4)	0.88	19.5 (5.6–88.3)	22.4 (7.2–47.2)	0.92	16.0 (2.2–26.5)	11.4 (1.2–35.8)	0.51
Neutrophils (% IR)	7.3 (3.0–21.0)	2.6 (0.6–14.2)	< 0.01	6.1 (3.0–14.6)	2.7 (0.7–14.5)	< 0.01	5.6 (0.0–21.0)	1.4 (0.0–12.0)	0.11
Eosinophils (cells/mm ²)	0.0 (0.0–3.0)	0.6 (0.0–15.1)	0.08	–	–	–	–	–	–
Basophils (cells/mm ²)	0.0 (0.0–1.9)	1.4 (0.0–7.7)	< 0.01	–	–	–	–	–	–

Table S4 Leucocyte infiltration in the alveolar parenchyma in healthy controls and asthmatic patients

Values are given as medians (range) unless otherwise stated.

Leucocyte	Controls (n = 8)	Asthma (n = 24)	P
T-helper cells (% IR)	0.63 (0.24–1.52)	2.18 (0.36–16.00)	< 0.01
T-cytotoxic cells (% IR)	4.44 (1.81–6.52)	5.75 (1.78–12.94)	0.26
B-cells (% IR)	0.75 (0.27–1.07)	0.60 (0.09–1.74)	0.48
NK cells (% IR)	2.74 (1.15–5.60)	3.99 (0.76–10.61)	< 0.05
Macrophages (% IR)	25.49 (16.76–40.68)	32.15 (13.92–62.12)	0.07
Neutrophils (% IR)	5.77 (3.55–11.21)	4.43 (2.03–16.47)	0.27
Eosinophils (cells/mm ²)	1.59 (0.0–2.85)	2.85 (0.0–10.19)	0.05
Basophils (cells/mm ²)	0.82 (0.0–1.94)	2.32 (0.0–6.80)	0.05

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