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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 (ICS). As conventional ICS have poor access to the peripheral airways, the aim of present study was to study the relationship between peripheral airway inflammation and clinical control in allergic asthma. Consequently, bronchial and transbronchial biopsies were obtained from poorly controlled asthmatics (n=12, Asthma Control Test (ACT) score <20), well-controlled asthmatics (n=12, ACT score ≥20) and healthy controls (n=8). Tissue sections were immunostained to assess multiple leukocyte populations. To determine the degree of T helper type 2 (Th2) immunity, the logarithmic value of the ratio between Th2 cells/mm^2 and Th1 cells/mm^2 was used as a surrogate score for Th2 skewed immunity. In the bronchi, the leukocyte infiltration pattern and the Th2-score were similar between well-controlled and poorly controlled asthmatics. In contrast, in the alveolar parenchyma the expression of T helper cells was significant higher in poorly controlled asthmatics compared to well-controlled asthmatics ($p<0.01$). Furthermore, the alveolar Th2-score was significantly higher in poorly controlled asthma (median 0.4) compared to the controlled patients (median -0.10, $p<0.05$). Additionally, in contrast to bronchial Th2-score, the alveolar Th2-score correlated significantly with ACT score ($r_s=-0.56$, $p<0.01$) in the pooled asthma group. Collectively, our data reveal an alveolar Th2-skewed inflammation specifically in asthma patients that are poorly controlled with ICS and suggest that pharmacological targeting of the peripheral airways may be beneficial in this large patient category.

INTRODUCTION

Asthma is a chronic inflammatory disorder characterized by airway obstruction and hyperresponsiveness to constricting stimuli. Inhaled corticosteroids (ICS) are the most effective anti-inflammatory medications to keep asthma under clinical control[1,2]. Yet, despite treatment with conventional ICS, 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 characterizing cellular inflammation and tissue remodelling in the bronchi of asthmatics. 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 suggest 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 asthmatics report inflammatory cell infiltrations both in small airways[7-10] as well as the alveolar parenchyma [7-9,11,12]. Due to the invasiveness required to characterize peripheral airway inflammation, most of these studies have involved patients with severe asthma, or asthma patients requiring lung resection for treatment of carcinoma. Therefore, the available data on peripheral inflammation in the less severe forms of asthma remains very scarce[13]. Of particular interest, from a therapeutic point of view, is to evaluate if peripheral airway inflammation is present in asthmatics that are poorly controlled with conventional ICS as these therapies have poor access to the lung periphery[14].

We have recently demonstrated that alveolar mast cells are dramatically altered in patients with poorly controlled atopic asthma compared to patients with allergic rhinitis or healthy control subjects[15,16]. Not only were the numbers of alveolar mast cells increased, infiltrating alveolar mast cells also displayed a marked increase in FcεRI-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 ICS have poor access. Consequently, with a focus on T helper cell profiles, the aim of present study was to, for the first time, reveal the detailed infiltration patterns of multiple infiltrating leukocyte populations in both bronchial and transbronchial biopsies in atopic asthma and 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 base-line conditions.

METHODS
A detailed description of the Methods is available in the online supplements, which is accessible online from this issue's table of content online at www.clinsci.org.

Subjects
The present study was approved by the ethics committee in Lund, Sweden (LU412-03) and all volunteers gave written and informed consent. The asthma cohort consisted of 24 patients who where 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 5 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 poorly controlled asthmatics and a cut-off score of 20 or above to identify well-controlled asthmatics[17]. 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 subjects included in present study were nonsmokers and had not had an upper respiratory tract infection within 3 weeks prior to medical examination. During the year before bronchoscopy, 2 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, 10 biopsies (5 bronchial and 5 transbronchial), were taken at the Department of Respiratory Medicine and Allergology, Lund University Hospital, Sweden during the period of May 2007 – March 2008 (healthy controls) and December 2008 – February 212 (asthmatics). For subjects that showed positive SPT to seasonal pollen, bronchoscopy was performed outside the pollen season. Bronchial biopsies 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 anesthesia with a flexible bronchoscope (IT160; Olympus Ltd, Tokyo, Japan) and transbronchial biopsies were taken with biopsy forceps (FB211D; Olympus Ltd.) 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 μm thick) from each tissue block were stained with Mayer's hematoxylin with the sole purpose to, in an unbiased manner, select 4 biopsies patient (2 bronchial and 2 transbronchial) 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 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 poorly controlled asthmatics, 2 bronchial and 2 transbronchial could be obtained from each patient. From the well-controlled asthmatics, 2 bronchial biopsies were selected from 11 out of the 12 patients and 2 transbronchial biopsies were selected from 9 out of the 12 patients. From each of the 3 remaining patient, at least 1 bronchial and 1 transbronchial high-quality biopsy was analyzed.

Immunohistochemistry

Immunohistochemistry was used to assess all major populations of infiltrating leukocytes. All antibodies used in present study (see Table E1 in the Online Supplements) 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 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, UK), which recognize a unique granule constituent of basophils[18] and antibodies to eosinophil cationic protein (clone EG2; Pharmacia-Upjohn Diagnostics AB, Sweden)[19] . Both of these antibodies have been used previously on bronchial biopsies[20-22]. To detect Th1 and Th2 cell, $CD4^+$ T_H cells were double stained with lineage specific transcription factors T-bet (to identify Th1 cells) and GATA-3 (to detect Th2 cells)[23]. The immunostaining was performed identically and simultaneously for all patients groups in an automated immunohistochemistry robot operating at room temperature (Autostainer LV-1; Dako A/S, Glostrup, Denmark).

Tissue analysis

Stained sections were digitally scanned in an automated digital slide-scanning robot (Scanscope CS, Aperio Technologies Inc, Vista, CA) operating with a 20x microscope lens and images were analyzed in ImageScope (v. 10.0.36.1805; Aperio Technologies Inc.). For single stained sections, a positive staining recognizing algorithm was set based on chromogen color and staining intensity for each leukocyte 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 leukocytes was determined by dividing the number of positive pixels with the total number of pixels, giving a percentage of immunoreactivity. In the bronchial biopsies, immunoreactivity was determined in the whole tissue section and intact epithelium. In the transbronchial biopsies, immunoreactivity was determined in the alveolar parenchyma. Eosinophils, basophils, Th1 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 per square millimeter 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 analyzed, the arithmetic mean of the two logarithmic values was used as the final score. In cases where one of the biopsies contained 0 Th1 or Th2 cells per square millimeter, a Th2-score of 1 (in case of 0 Th1 cells/mm^2) or -1 (in case of 0 Th2 cells/mm^2) was given.

Statistical Analysis

Data were analyzed in GraphPad Prism v .6 (GraphPad Software Inc., CA, USA) and values are given as median (range) unless otherwise stated. To detect significant differences between two groups, the nonparametric two-tailed Mann-Whitney test was. 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 asthmatics were atopics and all except two were airway hyperresponsive. Compared to well-controlled asthma, the poorly controlled asthma group was older (median 49 vs. 27) and had lower BMI (median 22.5 vs. 24.3). Pre-bronchodilator FEV_1 was lower in the poorly controlled asthma group, but the predicted value of pre-bronchodilator FEV_1 was similar between the poorly controlled (median 81.3%) and well-controlled asthma (median 86.0%). All asthmatics were treated with budesonide dry powder inhaler (BUD-DPI) except one well-controlled asthmatic that was treated with beclomethasone hydrofluoroalkane (BDP-HFA). The equivalent dose of BUD-DPI did not differ between the well-controlled (median 720 µg/day) and poorly controlled asthma (median 720 µg/day). The healthy control subjects were all non-atopics and lacked airway hyperresponsiveness (i.e. PD_{20} >2000 µg). All subjects in present study were non-smokers and only two individuals had a history of smoking (exsmokers of at least a year prior to biopsy).

Leukocyte infiltration patterns in the large airways

The expression of infiltrating leukocytes in the whole bronchial tissue compartment in well-controlled and poorly controlled asthma is presented in Figure 1 and example images of the different immunostained leukocyte populations are shown in Figure 2. Except for basophils, which tended to be higher in well-controlled asthmatics, the expression $CD4^+$ T helper cells, $CD8^+$ T cytotoxic cells, B cells, natural killer cells, macrophages, neutrophils and eosinophils was similar between well-controlled and poorly controlled asthma ($p\geq0.7$ for all outcomes (Fig 1). In the subepithelial compartment and intact epithelium, no significant differences were observed between the two asthma groups (see Table E2 in online supplements). In the comparison between healthy controls and the pooled asthma group, the asthma group had increased expression of $CD4^+$ T helper cells ($p<0.001$), natural killer cells ($p<0.05$), basophils ($p<0.01$) and lower expression of neutrophils ($p<0.01$) in the whole bronchial tissue compartment (see Table E3 online supplements).

Leukocyte infiltration pattern in the alveolar parenchyma

The alveolar expression of leukocytes in well-controlled and poorly controlled asthma is presented in Figure 3. The expression of $CD4^+$ T helper cells was significantly increased in poorly controlled asthma compared to well-controlled asthma ($p<0.01$). Basophils were few in numbers but significantly higher in well-controlled asthma ($p<0.05$). In comparison to healthy controls, the pooled asthma group was associated with increased expression of CD4+ T helper cells ($p<0.01$) and natural killer cells ($p<0.05$) (see Table E4 in online supplements). 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 asthmatics (see Table E4 in online supplements).

Bronchial and alveolar Th2-scores and correlations to clinical parameters

In the central airways, the Th2-score was similar between well-controlled (median 0.37) and poorly controlled asthma (median 0.22; p=0.3) (Fig 4A). In contrast, in the alveolar region, the Th2-score was significantly higher in poorly controlled asthma (median 0.4) compared to well-controlled asthma (median -0.10; p<0.05) (Fig 4B). In the pooled group of asthmatics, alveolar Th2-score correlated significantly with ACT score (r_s=-0.62, p<0.01) while bronchial Th2-score did not (r_s=0.03, p=0.9) (Fig 4C and 4D respectively). Alveolar Th2-score correlated also with FEV_1 (r_s=-0.63; p≤0.001) and FEV_1 % predicted (r_s=-0.43; p<0.05) while bronchial Th2-score did not (see Figure E1 in online supplements). Borderline significant correlations were found between both alveolar and bronchial T_H2-scores to PD_{20} (r_s=-0.42; p=0.05 and r_s=-0.39; p=0.07, respectively) in the pooled group of asthmatics that were airway hyperresponsive (n=22) (see Figure E1 in online supplements). In comparison to healthy controls, the bronchial Th2-score in the pooled group of asthmatics (median, 0.28) was similar to the controls (median 0.51, p=0.27) whereas the alveolar Th2-score in the pooled asthmatics (median 0.09) was higher compared to controls (median -0.15, p<0.05) (see Figure E2 in online supplements).

Th2-score in the individual biopsies
The bronchial and alveolar Th2-scores in the individual biopsies from healthy controls, well-controlled asthmatics and poorly controlled asthmatics is presented in Figure 5A and 5B respectively.

DISCUSSION
To the best of our knowledge, this is the first study that explores the pattern of infiltrating leukocytes and Th2 immunity in both bronchial and transbronchial biopsies from asthmatics with variable degree of clinical control and healthy control subjects. While our data show that the infiltration of leukocytes is virtually the same between well-controlled and poorly controlled asthmatics in the bronchial airways, the alveolar parenchyma in poorly controlled asthma is associated with increased expression CD4+ T helper cells and a Th2-biased immune profile. These data suggest that ICS control bronchial inflammation in both groups of asthmatics 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 that 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 IL-4, IL-5, IL-9 and IL-13[24]. In 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 asthmatics [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 asthmatics. Our main finding of a 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 as diesel exhaust may facilitate the deposition of allergen fragments into the alveolar region[28-30]. Whether such peripheral allergen deposition contribute to heightened alveolar T_H2 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 responses and mast cell activation[15]. In regard of potential alveolar mast cell activation, we recently discovered a significant expansion of FcεRI-

expressing mast cells with elevated surface-bound IgE in the alveolar parenchyma in uncontrolled asthmatics[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 neighboring conducting, or non-allergic Th2 triggers.

Despite the increased alveolar Th2-score in poorly controlled asthma, alveolar eosinophils were not increased compared to 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. While Th2 cells are long-lived and reside in the tissue, eosinophils 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 leukocytes 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 to mild-to-moderate asthmatics[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 asthmatics, which makes generalizability of the findings in present study an issue. However, due to the invasiveness required to sample transbronchial biopsies and the unique and exploratory nature of present study, we believe that the number of patients is appropriate in 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 poorly controlled asthmatics were older compared to the well-controlled asthmatics. 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 to patients between 18-64 years of age[36]. In the present study, none of the poorly controlled asthmatics were 65 years or older. It should also be mentioned that in a large European multicenter study where the mean age was 39.9 years, no associated between age and ACT score was found[37]. The mean age of the pooled group of asthmatics in our study was 37.7 years (data not shown). For those 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 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 ageing[38]. Although age-related changes of the immune function in human asthma is yet to be studied in detail, there are studies on 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 recently been reviewed[39] and are to include changes in neutrophils, NK 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 poorly controlled asthmatics. 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, this study suggest 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 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 that can be included, we were able to obtain statistically secured and clear results for all main parameters. While 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 that 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 inhaled corticosteroids mainly target the bronchial airways and have poor access to the peripheral airways.

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Figure legends and Tables

FIG 1. Scattergrams showing leukocyte infiltration in the bronchial airways in well-controlled and poorly controlled asthmatics. Each dot represents individual mean values and horizontal bars represent the median value for each patient group.

FIG 2. Bright field micrographs exemplifying the immunohistochemical staining of (**A**) CD4$^+$ Th cells, (**B**) CD8$^+$ T cytotoxic cells, (**C**) CD20$^+$ B cells, (**D**) CD57$^+$ natural killer cells, (**E**) CD68$^+$ Macrophages, (**F**) MPO$^+$ Neutrophils, (**G**) EG2$^+$ Eosinophil, (**H**) BB1$^+$ Basophil, (**I**) GATA3$^+$ and CD4$^+$ Th2 cells (arrows) and (**J**) T-Bet$^+$ and CD4$^+$ Th1 cells (arrows). In all cases the primary immunoreactivity is visualized with DAB 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 μm; B, D and E = 30 μm; F = 10 μm; G and I = 20 μm; and J = 15 μm.

FIG 3. Scattergrams showing leukocyte infiltration in the alveolar parenchyma in well-controlled and poorly controlled asthmatics. Each dot represents individual mean values and horizontal bars represent the median value for each patient group. *$p<0.05$, **$p<0.01$.

FIG 4. (A and B) Scattergrams showing Th2-scores in well-controlled and poorly controlled asthma in bronchial airways and alveolar parenchyma respectively. (**C and D**) Correlation between Th2-score and ACT score in the pooled group of asthmatics 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 well-controlled asthma patient that was treated with BDP-HFA. *$p<0.05$.
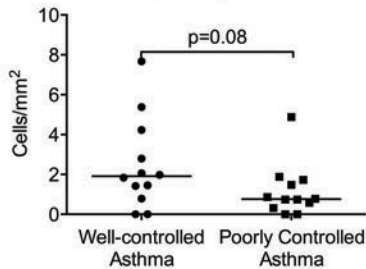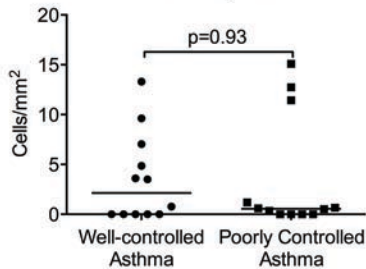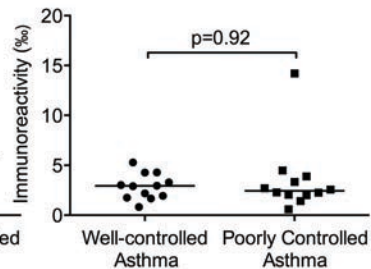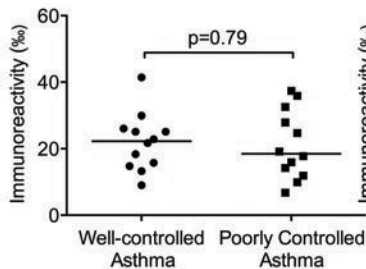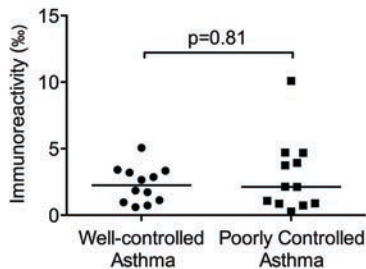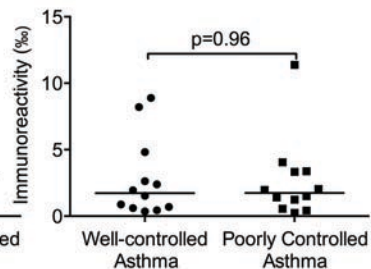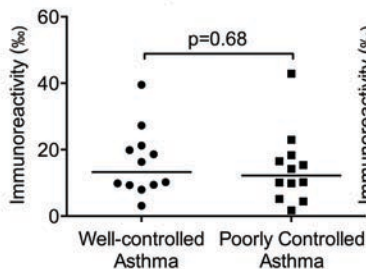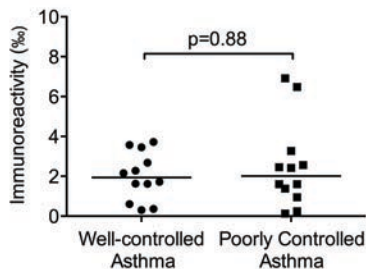
FIG 5. (A and B) Th2-scores in the individual biopsies from healthy controls, well-controlled and poorly controlled asthmatics in the bronchial and transbronchial biopsies respectively.
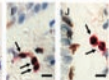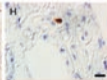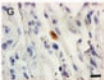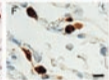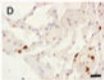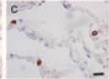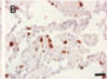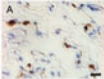
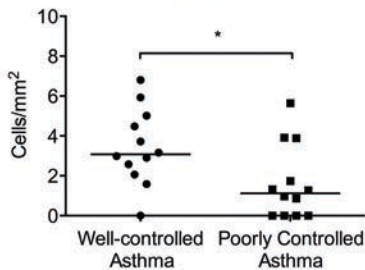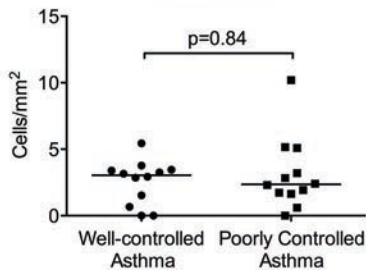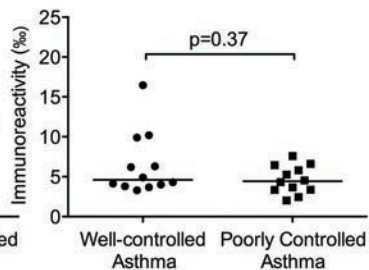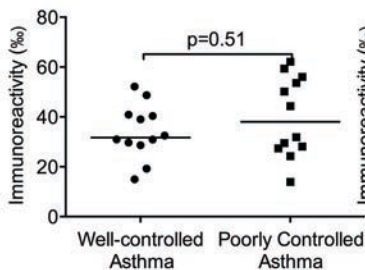**TABLE 1.** CLINICAL CHARACTERISTICS OF ASTHMA PATIENTS AND HEALTHY CONTROLS

	Healthy controls	Well-controlled asthma	Poorly controlled asthma	P^{*}
Sample size	8	12	12	-
Gender (M:F)	3:5	8:4	6:6	NS
Age (yrs)	23 (21-39)	27 (20-40)	49 (30-59)	<.0001
BMI (kg/m^2)	23.01 (19.38-27.10)	24.33 (20.55-29.40)	22.50 (19.59-24.97)	<.05
FEV$_1$ % pred.	98.05 (72.10-116.40)	85.95 (72.50-108.80)	81.25 (63.30-108.00)	NS
FEV$_1$ (L)	3.70 (2.38-5.38)	3.80 (2.35-5.15)	2.87 (1.84-4.64)	<.05
PD20 (μg)	2000†	773.0 (0.1-2000)	190.8 (0.1-1620)	NS
Atopy (Y/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
Neversmokers/Exsmokers	8/0	11/1	11/1	NS

*Well-controlled vs Poorly controlled asthma, †All patients had an unspecified PD20 value greater than 2000 μg.

Abbreviations: ACT = Asthma Control Test, BUD-DPI = Budesonide dry powder inhaler, FEV$_1$ = forced expiratory volume in 1 second, ICS = inhaled corticosteroids, NS = not significant, PD20 = provocative dose (metacholine) producing a fall in FEV$_1$ of 20 % or more.

T helper cells — p=0.88 — Well-controlled Asthma / Poorly Controlled Asthma — Immunoreactivity (‰)

T cytotoxic cells — p=0.68 — Well-controlled Asthma / Poorly Controlled Asthma — Immunoreactivity (‰)

B cells — p=0.96 — Well-controlled Asthma / Poorly Controlled Asthma — Immunoreactivity (‰)

Natural killer cells — p=0.81 — Well-controlled Asthma / Poorly Controlled Asthma — Immunoreactivity (‰)

Macrophages — p=0.79 — Well-controlled Asthma / Poorly Controlled Asthma — Immunoreactivity (‰)

Neutrophils — p=0.92 — Well-controlled Asthma / Poorly Controlled Asthma — Immunoreactivity (‰)

Eosinophils — p=0.93 — Well-controlled Asthma / Poorly Controlled Asthma — Cells/mm^2

Basophils — p=0.08 — Well-controlled Asthma / Poorly Controlled Asthma — Cells/mm^2

A

Th2-score

Bronchial biopsies

- Poorly controlled asthma
- Well-controlled asthma
- Healthy control

B

Th2-score

Transbronchial biopsies

- Poorly controlled asthma
- Well-controlled asthma
- Healthy control

Alveolar T Helper Type 2 Immunity in Atopic Asthma is Associated with Poor Clinical Control

Anders Bergqvist, Cecilia K Andersson, Michiko Mori, Andrew F Walls, Leif Bjermer, and Jonas S Erjefält.

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METHODS

Allergy screening

A standardized skin prick test (SPT; ALK-Abelló A/S, Copenhagen Denmark) was used on all subjects to screen for sensitization of 10 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.
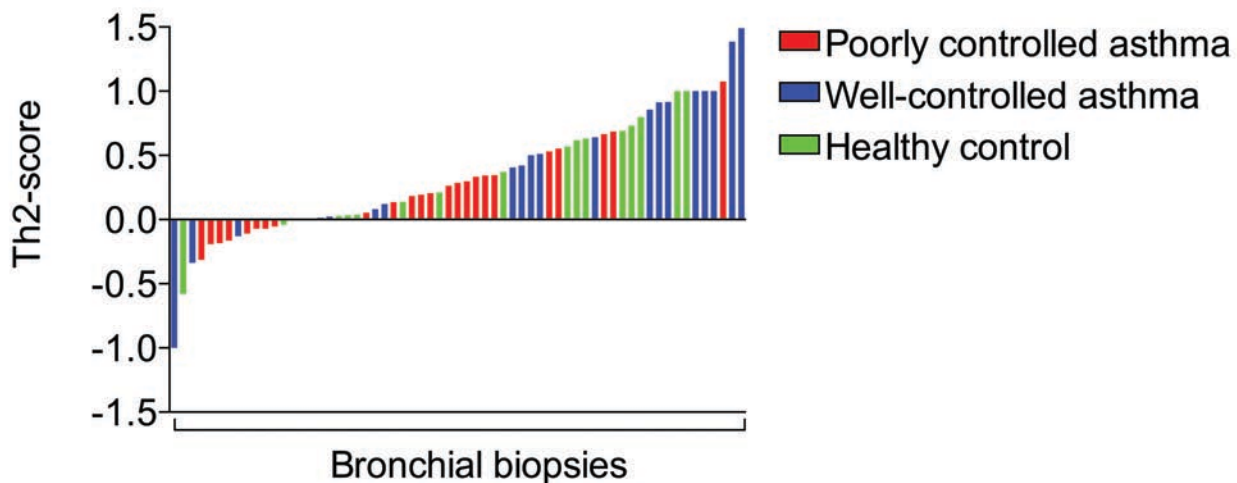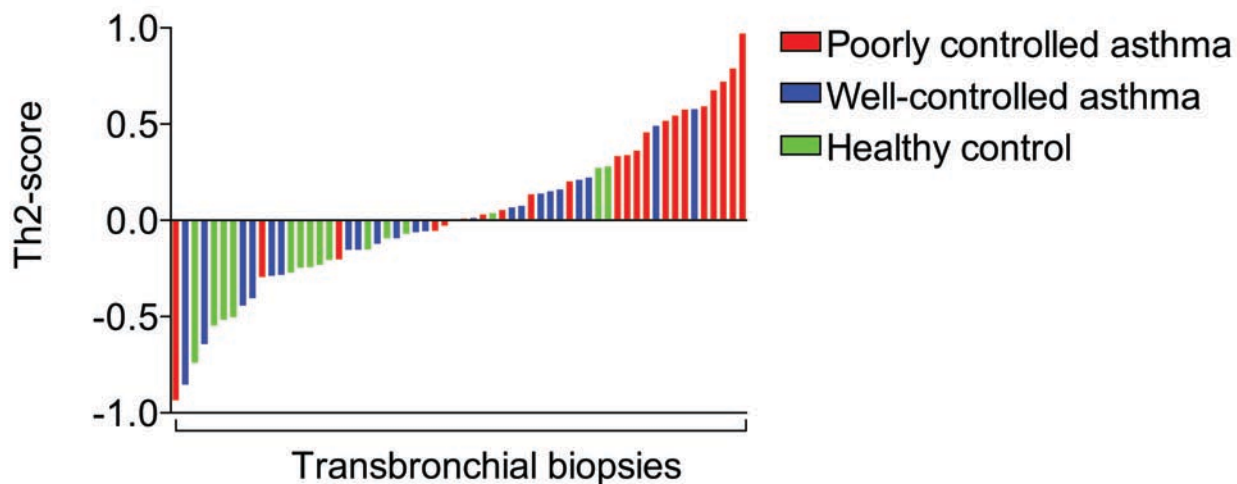
Spirometry and methacholine inhalation challenge

Lung function was measured with a MasterScope spirometer (v. 4.5; Erich Jaeger GmbH, Wurzburg, Germany) and reference values were obtained from Crapo et al[1]. Two FEV_1 values were recorded with less than 4% variation and the better value was recorded as the baseline. Bronchial hyperresponsiveness was measured with a methacholine inhalation challenge test performed with a tidal volume-triggered instrument (Aerosol Provocation System; Erich Jaeger GmbH.). The Aerosol Provocation System delivered a cumulative dose of 2000 μg methacholine in 5 increments (50, 150, 300, 600, and 900 μg) after an initial dose of 0.9% NaCl. A positive test was defined at the cumulative dose that caused the FEV_1 to decline by 20% or more from the baseline. When FEV_1 declined below 80% of the baseline value or when the total amount of methacholine (2000 μg) was delivered, 400 μg salbutamol was given to the subject. After 10 minutes, a new flow-volume spirometry was carried out to ensure proper patient recovery.

Tissue processing

From all subjects 5 bronchial and 5 transbronchial biopsies were collected and were immediately after excision placed in 4% buffered formaldehyde (fixation time, 1.5-2 hours at room temperature and 15-19 hours at 4℃), dehydrated and embedded in paraffin. Sections (3 μm thick) from each tissue block were stained with Mayer's hematoxylin 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 poorly controlled asthmatics, 2 bronchial and 2 transbronchial were selected from each patient. From the well-controlled asthmatics, 2 bronchial biopsies were selected from 11 out of the 12 patients and 2 transbronchial biopsies were selected from 9 out of the 12 patients. From each of the few remaining patient, 1 bronchial and 1 transbronchial high-quality biopsy were analyzed.

Single staining immunohistochemistry
Sections were incubated at 60 ºC for 20 minutes and then pretreated with heat-induced antigen retrieval in DIVA decloaker (Biocare Medical Inc, Concord, CA), pH 6, in a pressure cooker (2100 Retriever; Prestige Medical Ltd, Blackburn, UK) 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 A/S) according to manufacturer's instructions. In brief, sections were blocked with 0.3% hydrogen peroxide (Merck GmbH, Darmstadt, Germany) for 10 minutes, incubated with primary antibody (1 hour), incubated with a dextran polymer reagent containing peroxidase molecules and secondary antimouse and antirabbit antibodies (30 mins) and then finally incubated with non-permeable chromogen DAB (8 mins). Background staining was visualized with hematoxylin and sections were taken through graded alcohol baths (3 x 5 minutes) and xylene baths (3 x 5 minutes) before being mounted with Pertex mounting medium (Histolab Products AB, Gothenburg, Sweden).

Double staining immunohistochemistry
Sections were incubated at 60 ºC for 20 minutes and then pretreated (deparaffinized, rehydrated and antigen retrieved) in pH 9.0 using a PT Link (Dako A/S.). Sections were then stained either with T-Bet or GATA-3 antibodies using the EnVision™ Detection System Peroxidase/DAB, Rabbit/Mouse (K5007; Dako A/S) as described above. Next, a double staining blocking solution (Biocare Medical Inc.) was added (5 mins) to denature residual secondary antibodies on the dextran polymer in order to avoid cross-reactivity. Sections were also treated with 20% horse serum for 20 minutes (Vector Laboratories Inc, Burlingame, CA) and avidin/biotin blocking reagents (Dako A/S.) for 10 minutes respectively to avoid cross-reactivity. The sections were then incubated with antibodies towards CD4 for 1 hour and then with biotinylated horse anti-mouse secondary antibodies (Vector Laboratories Inc.) at a 1:200 dilution for 1 hour. After this, sections were treated with streptavidin-alkaline phosphatase solution (AbD Serotec Inc, Raleigh, NC) at 1:100 dilution for 40 minutes and finally with permanent red chromogen (Dako A/S.) at 1:100 dilution for 10 minutes. Background staining was visualized with hematoxylin and sections were dried at 60 ºC before taken through xylene baths (3 x 5 minutes) and finally mounted with Pertex mounting medium (Histolab Products AB.). Staining with matched isotype control antibodies for the T-Bet and GATA-3 antibodies resulted in no immunostaining.

References

E1. Crapo, R. O., Morris, A. H. and Gardner, R. M. (1981) Reference spirometric values using techniques and equipment that meet ATS recommendations. Am Rev Respir Dis. **123**, 659-664

Figure Legends

Fig E1. (A and B) Correlation between Th2-score and FEV_1 (L) in the pooled group of asthmatics in the bronchial airways and alveolar parenchyma respectively. **(C and D)** Correlation between Th2-score and FEV_1 % predicted in the pooled group of asthmatics in the bronchial airways and alveolar parenchyma respectively. **(E and F)** Correlation between Th2-score and PD_{20} in the pooled group of asthmatics that were airway hyperresponsive (i.e $PD_{20} < 2000$ µg) in the bronchial airways and alveolar parenchyma respectively. Each dot represents individual mean values.

Fig E2. (A and B) Bronchial and alveolar Th2-scores in healthy controls and the pooled group of asthmatics. Each dot represents individual mean values and the horizontal bar represents the median value for each respective group.

TABLE E1. PRIMARY ANTIBODIES USED IN IMMUNOHISTOCHEMISTRY					
Antibody	**Marker for**	**Species**	**Dilution**	**Clone/catalog #**	**Manufacturer**
CD4	Th cells	Mouse IgG1	1:100	1F6	Novocastra Ltd, Newcastle upon Tyne, UK
CD8	T cytotoxic cells	Mouse IgG1 kappa	1:640	C8/144b	Dako A/S, Glostrup, Denmark
CD20	B cells	Mouse IgG2a kappa	1:2000	L26	Dako A/S
CD57	Natural killer cells	Mouse IgM	1:200	NK-1	Novocastra
CD68	Macrophages	Mouse IgG3 kappa	1:1200	PG-M1	Dako A/S
MPO	Neutrophils	Rabbit polyclonal	1:20000	A0398	Dako A/S
EG2	Eosinophils	Mouse IgG1	1:1000	EG-2	Pharmacia-Upjohn Diagnostic AB, Sweden
BB1	Basophils	Mouse IgG2a	1:50	BB1	Andrew Walls Laboratory
T-BET	Th1 differentiated T_H cells	Rabbit polyclonal	1:4000	H-210	Santa Cruz Biotechnology Inc, San Diego, CA
GATA-3	Th2 differentiated T_H cells	Mouse IgG1 kappa	1:5000	L50-823	BD Pharmingen Inc, San Jose, CA
Isotype control	Non-human antigen	Mouse IgG1 kappa	1:1000	X0931	Dako A/S

Isotype control	Non-human antigen	Rabbit IgG	1:10	NP-0001	Epitomics Inc, Burlingame, CA

Abbreviations: T_H1 T helper type 1, T_H2 = T helper type 2. Ig = Immunoglobulin

TABLE E2. LEUKOCYTE INFILTRATION IN THE SUBEPITHELIUM AND INTACT EPITHELIUM IN WELL-CONTROLLED AND POORLY CONTROLLED ASTHMA

Leukocyte*	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
Natural killer 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. Values are given as median (range) unless otherwise stated.

TABLE E3. LEUKOCYTE INFILTRATION IN THE BRONCHIAL AIRWAYS IN HEALTHY CONTROLS AND ASTHMATICS

Leukocyte	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
Natural killer 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/mm²)	0.0 (0.0-3.0)	0.6 (0.0-15.1)	0.08	-	-	-	-	-	-
Basophils (cells/mm²)	0.0 (0.0-1.9)	1.4 (0.0-7.7)	**<0.01**	-	-	-	-	-	-

Abbreviations: IR = immunoreactivity. Values are given as median (range) unless otherwise stated.

TABLE E4. LEUKOCYTE INFILTRATION IN THE ALVEOLAR PARENCHYMA IN HEALTHY CONTROLS AND ASTHMATICS

Leukocyte	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
Natural killer 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

Abbreviations: IR = immunoreactivity. Values are given as median (range) unless otherwise stated.

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