

Mast cell–associated alveolar inflammation in patients with atopic uncontrolled asthma

Cecilia K. Andersson, PhD,^a Anders Bergqvist, MSc,^a Michiko Mori, MSc,^b Thais Mauad, MD, PhD,^c Leif Bjerner, MD, PhD,^a and Jonas S. Erjefält, PhD^{a,b} Lund, Sweden, and São Paulo, Brazil

Background: A significant proportion of patients with asthma have persistent symptoms despite treatment with inhaled glucocorticosteroids.

Objective: We hypothesized that in these patients, the alveolar parenchyma is subjected to mast cell–associated alterations.

Methods: Bronchial and transbronchial biopsies from healthy controls (n = 8), patients with allergic rhinitis (n = 8), and patients with atopic uncontrolled asthma (symptoms despite treatment with inhaled glucocorticosteroids; mean dose, 743 µg/d; n = 14) were processed for immunohistochemical identification of mast cell subtypes and mast cell expression of FcεRI and surface-bound IgE.

Results: Whereas no difference in density of total bronchial mast cells was observed between patients with asthma and healthy controls, the total alveolar mast cell density was increased in the patients with asthma ($P < .01$). Division into mast cell subtypes revealed that in bronchi of patients with asthma, tryptase positive mast cells (MC_T) numbers decreased compared with controls ($P \leq .05$), whereas tryptase and chymase positive mast cells (MC_{TC}) increased ($P \leq .05$). In the alveolar parenchyma from patients with asthma, an increased density was found for both MC_T ($P \leq .05$) and MC_{TC} ($P \leq .05$). The increased alveolar mast cell densities were paralleled by an increased mast cell expression of FcεRI ($P < .001$) compared with the controls. The patients with asthma also had increased numbers ($P < .001$) and proportions ($P < .001$) of alveolar mast cells with surface-bound IgE. Similar increases in densities, FcεRI expression, and surface-bound IgE were not seen in separate explorations of alveolar mast cells in patients with allergic rhinitis.

Conclusion: Our data suggest that patients with atopic uncontrolled asthma have an increased parenchymal infiltration of MC_T and MC_{TC} populations with increased expression of

FcεRI and surface-bound IgE compared with atopic and nonatopic controls. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Mast cells, asthma, FcεRI, IgE, allergy, peripheral inflammation, alveolar parenchyma

Asthma is a chronic inflammatory airway disease that is characterized by a reversible airway obstruction and airway hyperreactivity.^{1,2} Most patients have an allergic component in which IgE plays a central role by activating key immune cells through the high-affinity IgE receptor, FcεRI.^{3,4} Although treatment with bronchodilators and inhaled glucocorticosteroids (ICSs) generally provide good control of the disease, a significant proportion of patients with asthma have persistent symptoms despite conventional therapy.⁵ This phenomenon, referred to as *uncontrolled asthma*,^{6,7} represents a major challenge for improved asthma control.

Despite the clinical significance of uncontrolled asthma, little is known about the inflammatory processes that evoke symptoms in this group of patients. One possibility is existence of steroid-resistant inflammatory components in the central airways.⁸ Another alternative is involvement of peripheral airways,^{9,10} which are difficult to reach by conventional inhalation therapy.¹¹ The few previous studies that have explored transbronchial biopsies from patients with asthma provide clear indications that both small airways and alveolar tissues may be subjected to cellular inflammation in asthma.¹²⁻¹⁴

Mast cells have long been recognized as a key cell of the allergic reaction in atopic asthma by virtue of their expression of FcεRI.¹⁵ They are widely present in high numbers in human peripheral airways, including the alveolar region.^{10,12,16-18} We recently identified a distinct mast cell population in the alveolar tissue of normal lungs.¹⁹ These poorly studied alveolar mast cells, which are characterized by a low FcεRI expression, make up a large mast cell population in the human lung.^{12,19,20}

Increased numbers of bronchial mast cells and elevated levels of IgE have been described in allergic asthma.^{10,12,17,21,22} From this and the fact that high IgE levels may lead to increased FcεRI expression on mast cells,^{23,24} we hypothesized that patients with ICS-treated, atopic, uncontrolled asthma have a significantly altered mast cell population in which the normally FcεRI low-expressing alveolar mast cells have acquired an FcεRI-expressing phenotype. To test this hypothesis, mast cell densities and mast cell expression of FcεRI and surface-bound IgE were analyzed in bronchial and transbronchial biopsies from patients with atopic, uncontrolled asthma and healthy control subjects. Separate comparisons were also made with patients with atopic allergic rhinitis (AR) with no concomitant asthma.

From the Departments of ^aRespiratory Medicine and Allergology and ^bExperimental Medical Science, Lund University; and ^cthe Department of Pathology, São Paulo University.

Supported by the Heart and Lung Foundation, Sweden; the Swedish Medical Research Council; the Swedish Asthma and Allergy Associations Research Foundation; and the Crafoord Foundation.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

Received for publication June 11, 2010; revised December 22, 2010; accepted for publication January 12, 2011.

Reprint requests: Jonas S. Erjefält, PhD, Professor, Airway Inflammation Unit, Department of Experimental Medical Science, BMC D12, Lund University, SE-22184, Lund, Sweden. E-mail: jonas.erjefalt@med.lu.se.

0091-6749/\$36.00

© 2011 American Academy of Allergy, Asthma & Immunology
doi:10.1016/j.jaci.2011.01.022

Abbreviations used

ACT:	Asthma control test
AR:	Allergic rhinitis
CF:	Cystic fibrosis
COPD:	Chronic obstructive pulmonary disease
ICS:	Inhaled glucocorticosteroid
MC _T :	Tryptase-positive mast cell (mucosal mast cell)
MC _{TC} :	Tryptase and chymase-positive mast cell (connective tissue mast cell)
1% PLP:	Periodate-lysine containing 1% paraformaldehyde
SPT:	Skin prick test

METHODS**Subjects**

Patients with atopic uncontrolled asthma, nonatopic and atopic control groups. The current study involved 14 non-smoking patients with uncontrolled atopic asthma according to Global Initiative for Asthma guidelines and an asthma control test (ACT).^{6,25} Eight healthy never-smoking nonatopic subjects who had a negative skin prick test (SPT) result, were not hyperresponsive to methacholine, and lacked any history of respiratory symptoms were used as controls. A separate control group, representing atopy without asthma, included 8 patients with clinically confirmed AR.²⁶

From each of the 30 subjects, 5 central airway biopsies and 5 transbronchial biopsies (in total, 300 biopsies) were collected during a study period from November 2005 to June 2010 at the Department of Respiratory Medicine and Allergology, Lund University Hospital (for methodologic details, see reference²⁷ and the Bronchoscopy and collection of bronchial and transbronchial biopsies section in the Online Repository at www.jacionline.org). All subjects gave their written informed consent to participate in the study, which was approved by the ethics committee in Lund (LU412-03).

Lungs from patients with advanced stages of chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) were included to study the FcεRI expression on alveolar mast cells in lungs subjected to nonallergic inflammation. The clinical characterization of these patients and their matching controls are presented in this article's Table E1 and the Subjects section in the Online Repository at www.jacionline.org.

Allergy screening

A standardized SPT (ALK-Abelló, Copenhagen, Denmark) was performed on all subjects in all cohorts (controls, asthma, AR, COPD, and CF) and was used to screen for sensitization for 10 aeroallergens (birch, timothy, mugwort, cat, dog, horse, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Aspergillus fumigatus*, and *Cladosporium herbarum*). Atopy was defined as a positive SPT (weal reaction larger or equal to histamine positive control) to 1 or more allergens. Patients with positive SPT to pollen without any other sensitivity were classified as seasonal, whereas patients with multiple sensitivities (pollen, animal, mold, and/or mite) were classified as perennial. For all subjects with positive SPT to seasonal pollen, bronchoscopy procedures were performed outside pollen season.

Tissue processing

Bronchial and transbronchial biopsies. All biopsies from patients with uncontrolled asthma and 4 of 5 bronchial and 4 of 5 transbronchial biopsies from the healthy controls were immediately placed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin. Serial sections from all paraffin blocks were stained with Mayer hematoxylin, and these were used to select 2 bronchial and 2 transbronchial biopsies from each patient that had a well preserved morphology and were without any crush, or mechanically induced stretch artifacts. The selected biopsies were used for quantification of mast cell-related parameters.

The remaining biopsies from the control patients and the rhinitis cohort were immersed in periodate-lysine containing 1% paraformaldehyde (1%

PLP) for 4 hour at 4°C. Specimens were embedded in optimal cutting temperature medium (Tissue-Tek, Miles Laboratories, Ind) and frozen. Serial cryosections from all biopsies were generated and stored until histologic assessments.

Immunohistochemistry

All antibodies used have been extensively validated for staining of paraffin-embedded human tissue sections (and cryosections) in research and routine clinical diagnosis (see this article's Table E2 in the Online Repository at www.jacionline.org). For details on immunohistochemical protocols and specificity controls,^{19,20} see Methods section in the Online Repository. Staining was absent in sections using isotype-matched control antibodies (Dako, Glostrup, Denmark). Staining was performed identically for all patient groups.

Double immunohistochemical staining of MC_{TC} and MC_T. A double staining protocol was used for simultaneous visualization of tryptase and chymase positive mast cells (MC_{TC}) and tryptase positive mast cells (MC_T) cells.^{18-20,28-32} The immunohistochemistry protocol was performed by using an automated immunohistochemistry robot (Autostainer; Dako) with EnVision G2 Doublestain System (K5361, Dako); for protocol details, see Table E2 and the Double immunohistochemical staining of MCTC and MCT section in the Online Repository.

Immunohistochemical identification of FcεRI⁺ and IgE⁺ mast cells. A triple staining immunofluorescence protocol^{19,20,33,34} was used to visualize both MC_{TC} and MC_T populations simultaneously together with their expression of the IgE receptor (FcεRI) or surface-bound IgE (see the Immunohistochemical identification of FcεRI1 and IgE1 mast cells section in the Online Repository and Table E2).

Histologic analysis and quantification**Quantification of densities of mast cell subtypes.**

High-resolution images of sections stained for MC_{TC} and MC_T were generated through a ×20 microscope lens by an automated digital slide-scanning robot (Scanscope CS; Aperio, Vista, Calif). In bronchial biopsies, the densities of MC_T and MC_{TC} were calculated in subanatomic compartments: epithelium, subepithelial tissue (excluding smooth muscle and glands), smooth muscle tissue, and subepithelial glands. An image analysis program (ImageScope, v10.0.36.1805; Aperio) calculated the tissue area within the delineated region by automatically excluding any nontissue regions (ie, regions without any tissue components), and the proper tissue density (expressed as cells/mm² tissue) was calculated for each mast cell subtype.^{14,19,20,30,35-37}

Quantification of FcεRI⁺ and IgE⁺ mast cells. After triple immunofluorescence staining, the filter setting was adjusted to reveal the tryptase-positive mast cells at 488 nm. By alternating the filter settings, each tryptase-positive cell was examined for the presence of chymase (647 nm) as well as expression of FcεRIα or surface-bound IgE (555 nm). The density of mast cells expressing FcεRI and IgE was calculated by multiplying the percentage of mast cells positive for FcεRI (MC^{FcεRI+}) or mast cells with bound IgE (MC^{IgE+}) with the total mast cell density in the same tissue region (for further details and quantification on COPD and CF, see reference^{19,20} and the Tissue analysis section in the Online Repository).

Statistical analysis

Data were analyzed statistically on mean values from each patient by using the Mann-Whitney rank-sum test for comparison between 2 groups (disease vs control) and GraphPad Prism v. 5 (GraphPad Software Inc, La Jolla, Calif). For all outcomes, a *P* value ≤.05 was considered significant (**P* ≤.05; ***P* <.01; ****P* <.001).

RESULTS**Clinical characteristics**

An overview of the patient characteristics is presented in Table I.

Uncontrolled asthma. The 14 patients with asthma included in the study had symptomatic uncontrolled asthma (ACT

TABLE I. Subject characteristics for uncontrolled asthma, AR, and healthy controls

Subject	Asthma/rhinitis/controls	Sex (M/F)	Age (y)	FEV ₁ (L)	FEV ₁ % of pred	PD ₂₀ (μg)	Atopy (Y/N)	Rhinitis (Y/N)	ICS/d (μg)	ACT score	Smoking (Y/N)
1	Control	M	29	5.11	116.4	>2000	N	N	0	NA	N
2	Control	F	23	3.68	95.4	>2000	N	N	0	NA	N
3	Control	M	39	4.91	109.8	>2000	N	N	0	NA	N
4	Control	M	23	5.38	113.5	>2000	N	N	0	NA	N
5	Control	F	23	3.71	100.7	>2000	N	N	0	NA	N
6	Control	F	22	2.38	72.1	>2000	N	N	0	NA	N
7	Control	F	23	3.53	95.1	>2000	N	N	0	NA	N
8	Control	F	21	3.25	89.4	>2000	N	N	0	NA	N
9	Rhinitis	M	22	3.98	108.7	>2000	Y (p)	Y	0*	NA	N
10	Rhinitis	F	25	2.98	96.3	>2000	Y (p)	Y	0	NA	N
11	Rhinitis	F	36	3.21	103.8	>2000	Y (p)	Y	0	NA	N
12	Rhinitis	M	59	3.01	138.0	>2000	Y (p)	Y	0*	NA	N
13	Rhinitis	F	24	3.42	96.0	>2000	Y (p)	Y	0	NA	N
14	Rhinitis	F	24	3.21	105.0	>2000	Y (p)	Y	0	NA	N
15	Rhinitis	M	29	4.69	114.6	>2000	Y (s)	Y	0	NA	N†
16	Rhinitis	F	31	3.68	109.3	>2000	Y (p)	Y	0	NA	N
17	Asthma	M	45	4.25	104.2	243.4	Y (p)	Y	400	13	N
18	Asthma	M	59	3.17	81.0	672.0	Y (p)	Y	800	19	N
19	Asthma	M	27	3.86	81.7	>2000	Y (p)	N	800	20	N
20	Asthma	M	22	3.58	79.9	251.5	Y (p)	Y	400	21	N
21	Asthma	M	58	2.80	63.3	68.0	Y (p)	Y	400	13	N
22	Asthma	M	30	4.01	90.1	1620.0	Y (p)	Y	800	18	N
23	Asthma	F	50	2.14	74.7	69.3	Y (p)	Y	800	16	N
24	Asthma	F	24	2.35	72.5	381.9	Y (s)	Y	1200	20	N
25	Asthma	F	50	2.07	72.3	138.2	Y (p)	Y	800	17	N‡
26	Asthma	F	52	2.37	81.5	279.1	Y (p)	Y	800	14	N
27	Asthma	M	38	4.64	108.0	541.0	Y (p)	Y	1200	17	N
28	Asthma	M	37	4.14	96.0	500.0	Y (p)	Y	400	18	N§
29	Asthma	M	25	3.35	73.3	59.5	Y (p)	Y	800	18	N
30	Asthma	F	42	2.55	81.8	326.0	Y (s)	Y	800	11	N

F, Female; M, male; N, no; NA, not applicable; p, perennial; pred, predicted; s, seasonal; Y, yes.

*Nasal corticosteroid as needed.

†Exsmoker since 2003.

‡Exsmoker since 1985.

§Exsmoker since 2001.

score ranging 11-21). All were atopic (ie, positive SPT), and all but 1 had rhinitis. All patients with asthma were treated with ICSs (budesonide) and inhaled bronchodilators (Table I). Two patients were treated with leukotriene receptor antagonists, and 3 had nasal corticosteroids and antihistamines as needed. In addition, 1 patient was treated for hypertension (losartan potassium/hydrochlorothiazide), 2 for gastritis (omeprazole), and 1 with vitamin B substitute. None of the patients was treated with anti-IgE therapy. Two patients had seasonal and 12 had perennial allergy.

AR. The 8 patients with AR with no concomitant asthma all had positive SPT and were not hyperresponsive to methacholine (PD₂₀ > 2000 μg). None of the patients with AR were treated with inhaled bronchodilators or glucocorticosteroids. Two had nasal corticosteroids and 4 had antihistamines as needed. One patient had seasonal and 7 had perennial allergy.

Healthy controls. All healthy controls were without any respiratory symptoms, had normal lung function, and had a negative SPT and methacholine challenge test (PD₂₀ > 2000 μg). FEV₁ % predicted was lower in patients with asthma (81 [63-108] FEV₁ % predicted) compared with healthy controls (98 [72-116] FEV₁ % predicted; *P* = .03) and patients with rhinitis (107 [96-138] FEV₁ % predicted; *P* = .001). No difference in FEV₁ % predicted was found between controls and rhinitis (*P* = .3). In addition, 5 patients with CF and 10

patients with COPD were investigated^{19,20} (for patient and protocol details, see Methods and Results sections in the Online Repository).

Characterization of mast cell phenotypes in uncontrolled asthma and healthy controls

Densities of MC_T and MC_{TC} populations. In central airways, the total tissue density of mast cells did not differ between patients with uncontrolled asthma and healthy control subjects (Table II). In contrast, the alveolar parenchyma displayed increased numbers of mast cells in patients with uncontrolled asthma compared with healthy controls (Table II). The unaltered total mast cell numbers in central airways in uncontrolled asthma was a result of a decrease in MC_T numbers combined with an increase in MC_{TC} numbers compared with healthy controls (Table II; Fig 1). The significant increase in total alveolar mast cell numbers in patients with uncontrolled asthma was a result of an increase in both MC_T cells and MC_{TC} numbers compared with healthy controls (Table II; Fig 1).

Microlocalization of mast cell subtypes in central airways. The highest density of mast cells was found in the lamina propria for both control subjects and patients with asthma. No difference in the distribution of mast cells was found for the

TABLE II. Mast cell densities and expression of FcεRI and mast cell-bound IgE in patients with uncontrolled asthma and AR

		Central airways			Alveolar parenchyma		
		Controls (n = 8)	Uncontrolled asthma (n = 14)	P value	Controls (n = 8)	Uncontrolled asthma (n = 14)	P value
Uncontrolled asthma density (per mm ²)*							
	Total	69 (16-185)	40 (12-109)	.2	53 (0-241)	210 (59-591)	.006
	MC _T	60 (11-179)	19 (0-69)	.05	33 (0-239)	178 (22-456)	.01
	MC _{TC}	10 (0-24)	24 (5-83)	.05	12 (0-31)	38 (5-299)	.04
Expression (%)	FcεRI	69 (43-100)	86 (50-100)	.1	3 (0-11)	81 (8-100)	.0002
	IgE	31 (11-75)	91 (30-100)	.003	0 (0-12)	73 (13-100)	.0001
AR Density (per mm ²)†		Controls (n = 8)	AR (n = 8)	P value	Controls (n = 8)	AR (n = 8)	P value
	Total	79 (31-155)	83 (65-319)	.7	61 (0-179)	79 (32-186)	.2
	MC _T	72 (10-155)	66 (14-265)	1.0	46 (0-149)	64 (0-180)	.6
	MC _{TC}	15 (0-28)	21 (8-61)	.3	10 (0-30)	18 (6-68)	.2
Expression (%)	FcεRI	73 (40-100)	76 (40-100)	.6	0 (0-29)	0 (0-19)	.8
	IgE	13 (0-50)	30 (0-51)	.2	0 (0-17)	11 (0-37)	.02

Data are presented as medians (ranges). *n*, Number of patients in the group. A mean value per each patient was calculated from 2 bronchial and 2 transbronchial biopsies, respectively. The differences between the control group and disease group were then calculated by using the Mann-Whitney test. Results are considered significant for $P \leq .05$. Results not significant are shown in italics.

*Paraformaldehyde-fixed, paraffin-embedded tissue.

†PLP-fixed cryo tissue.

MC_T subclass in patients with asthma compared with controls (see this article's [Table E3](#) in the Online Repository at www.jacionline.org). The MC_{TC} density increased in the smooth muscle layer in patients with asthma (7.1 [0-25] mast cells per mm²) compared with controls (1.0 [0-5] mast cells per mm²; $P = .01$; [Table E3](#)).

Expression of FcεRIα and IgE on bronchial and alveolar mast cells. Both FcεRIα and IgE immunoreactivity displayed a characteristic membrane staining in triple-stained immunofluorescence sections. As previously shown,^{12,19,38} the proportion of mast cells expressing FcεRIα was high in central airways in healthy subjects, and no significant difference in expression compared with patients with uncontrolled asthma was observed ([Fig 2, A](#); [Table II](#)). The mast cell expression of FcεRIα did not differ between controls and patients with asthma for MC_T ($P = .4$) or for the MC_{TC} subtype ($P = .5$). In contrast, in the alveolar parenchyma, the mast cell expression of FcεRIα was low in healthy controls and significantly higher in uncontrolled asthma ([Fig 2, B-D](#); [Table II](#)). The increased FcεRIα expression in uncontrolled asthma was further confirmed by using a computerized image analysis approach to calculate the area of FcεRIα immunoreactivity on individual mast cells (see the Quantification of FcεRII and IgE1 mast cells section in the Online Repository).

In central airways, the proportion of IgE⁺ mast cells was low in healthy controls and significantly increased in patients with uncontrolled asthma ([Fig 2, E](#); [Table II](#)). Also in the alveolar parenchyma, the proportion of IgE⁺ mast cells was low in controls and significantly increased ([Fig 2, F-H](#); [Table II](#)). As for the expression of FcεRIα, no difference in mast cell-bound IgE was found between MC_T and MC_{TC} subclasses in central airways (controls, $P = .5$; asthma, $P = .2$) or in the alveolar parenchyma (controls, $P = .4$; asthma, $P = .1$).

In the alveolar parenchyma, the tissue density of FcεRIα-positive mast cells was increased in patients with uncontrolled asthma (132 [9-591] MC^{FcεRIα+}/mm²) compared with controls (1.5 [0-27] MC^{FcεRIα+}/mm²; $P = .0003$; [Fig 3, A](#)). Also, an increase in the density of mast cells positive for surface-bound IgE was found in the patients with asthma (133 [8-591] MC^{IgE+}/mm²) compared with

controls (0 [0-3] MC^{IgE+}/mm²; $P = .0001$; [Fig 3, B](#)). For correlation of mast cell parameters and patient lung function, see [Table E4](#) in the Online Repository at www.jacionline.org.

FcεRIα and IgE expression on alveolar mast cells in AR and nonallergic inflammatory diseases

AR. In AR, no significant change in total mast cell numbers or the density of MC_T and MC_{TC} was found in central airways or in alveolar parenchyma compared with healthy controls ([Table II](#)). No increase in the proportion of mast cells expressing the FcεRIα could be found in central airways or in the alveolar parenchyma in patients with AR compared with controls ([Table II](#)). No significant increase in the proportion of mast cells with surface-bound IgE was found in central airways. However, an increase in the proportion of mast cells with surface-bound IgE was found in the alveolar parenchyma in patients with AR compared with controls ([Table II](#)).

Nonallergic lung diseases: Comparison with COPD and CF. The FcεRIα expression was high in central airways in controls compared with the same compartment in COPD and CF. In the alveolar parenchyma, the mast cell FcεRIα expression was low in controls; no significant change was found in patients with COPD or CF (see the Mast cell expression of FcεRIα in nonallergic lung diseases: comparison with COPD and CF section in the Online Repository).

DISCUSSION

Accumulated evidence from physiological studies and tissue explorations suggests that inflammatory processes in the distal airways contribute to asthma pathogenesis.³⁹ The current study advances our insight about the nature of this inflammation by identifying an alveolar infiltration of altered MC_T and MC_{TC} populations as a novel histopathological feature.

The current study took advantage of our possibility to obtain bronchial as well as transbronchial biopsies, not only from patients with uncontrolled asthma, but also from healthy control subjects. Thus, our approach allowed the first exploration of how

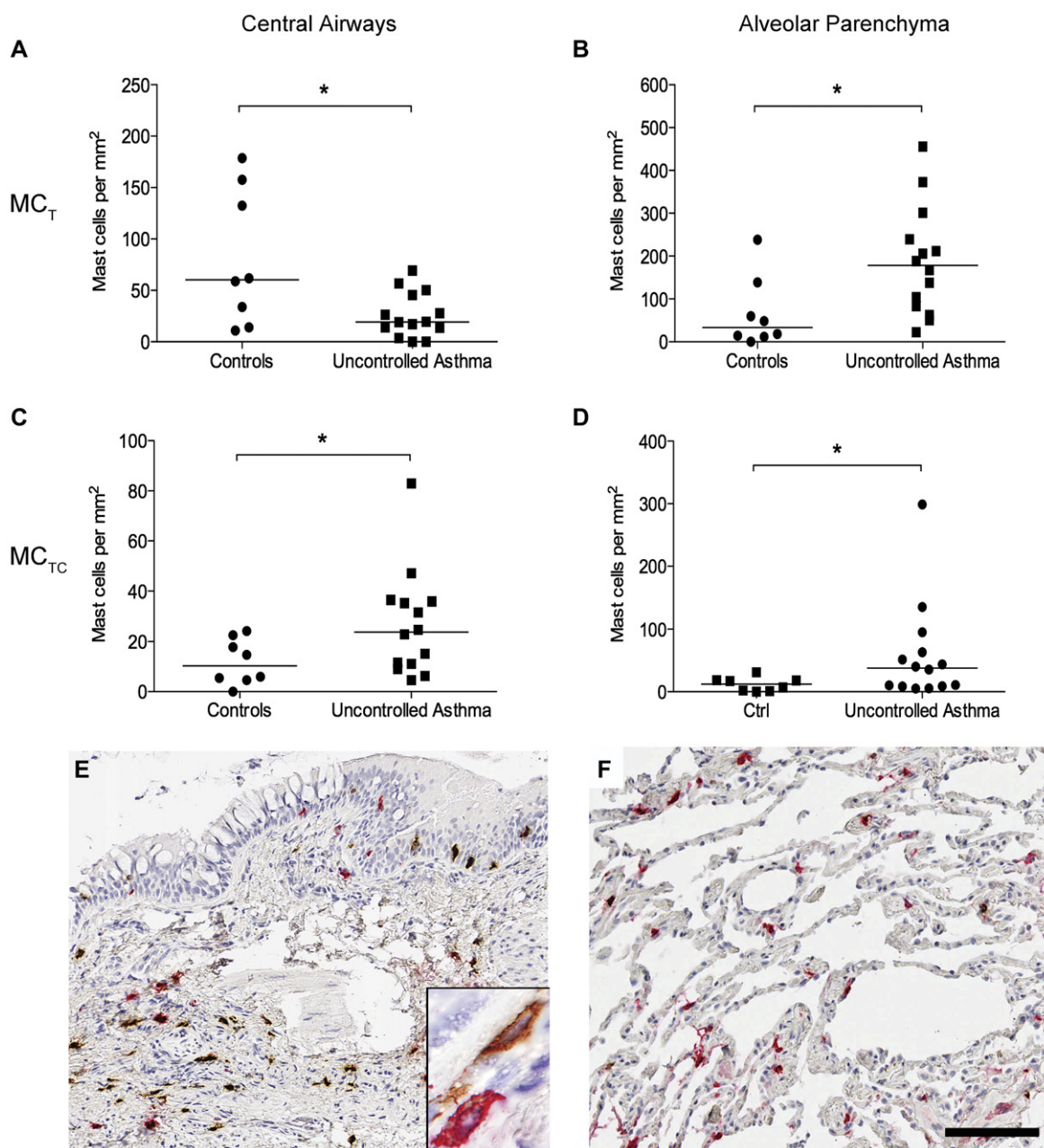


FIG 1. MC_T and MC_{TC} in central airways (**A and C**) and alveolar parenchyma (**B and D**) in uncontrolled asthma compared with healthy controls. **E**, Central airways; **F**, alveolar; representative micrographs from patients with asthma, double-stained for MC_T and MC_{TC}. Scale bar, **E** and **F** = 100 μ m. Inset in **E** is a close-up image ($\times 600$) of neighboring MC_{TC} and MC_T cells. Horizontal bars indicate median value.

mast cells in both bronchial and alveolar compartments in patients with uncontrolled asthma differ from healthy baseline conditions.

The discovery of increased Fc ϵ RI expression on alveolar mast cells in uncontrolled asthma represents a major finding in this study. Mast cells in most types of tissues, especially those facing the external environment, have a high basal expression of Fc ϵ RI.¹⁵ The alveolar mast cells, however, have under healthy conditions a very low Fc ϵ RI expression.¹⁹ In this study, the number of Fc ϵ RI-expressing mast cells in the alveolar parenchyma in uncontrolled asthma was 40-fold higher than what could be observed in healthy controls. Furthermore, this was associated with an equally robust (500-fold) increase in numbers of mast

cells with surface-bound IgE, suggesting that the alveolar mast cells in uncontrolled asthma have acquired a phenotype fully capable of classic IgE-mediated activation. Importantly, this feature seems to be specific to asthma because by same staining techniques, we could not detect similar changes in patients with severe nonallergic alveolar inflammation (end-stage CF or COPD) or atopic patients with AR.

The low expression of Fc ϵ RI α on alveolar mast cells in AR present in our study indicates that atopy *per se* does not cause the altered alveolar mast cell phenotypes found in uncontrolled asthma. It should be noted, however, that the situation may be different in patients with AR during episodes of increased allergen

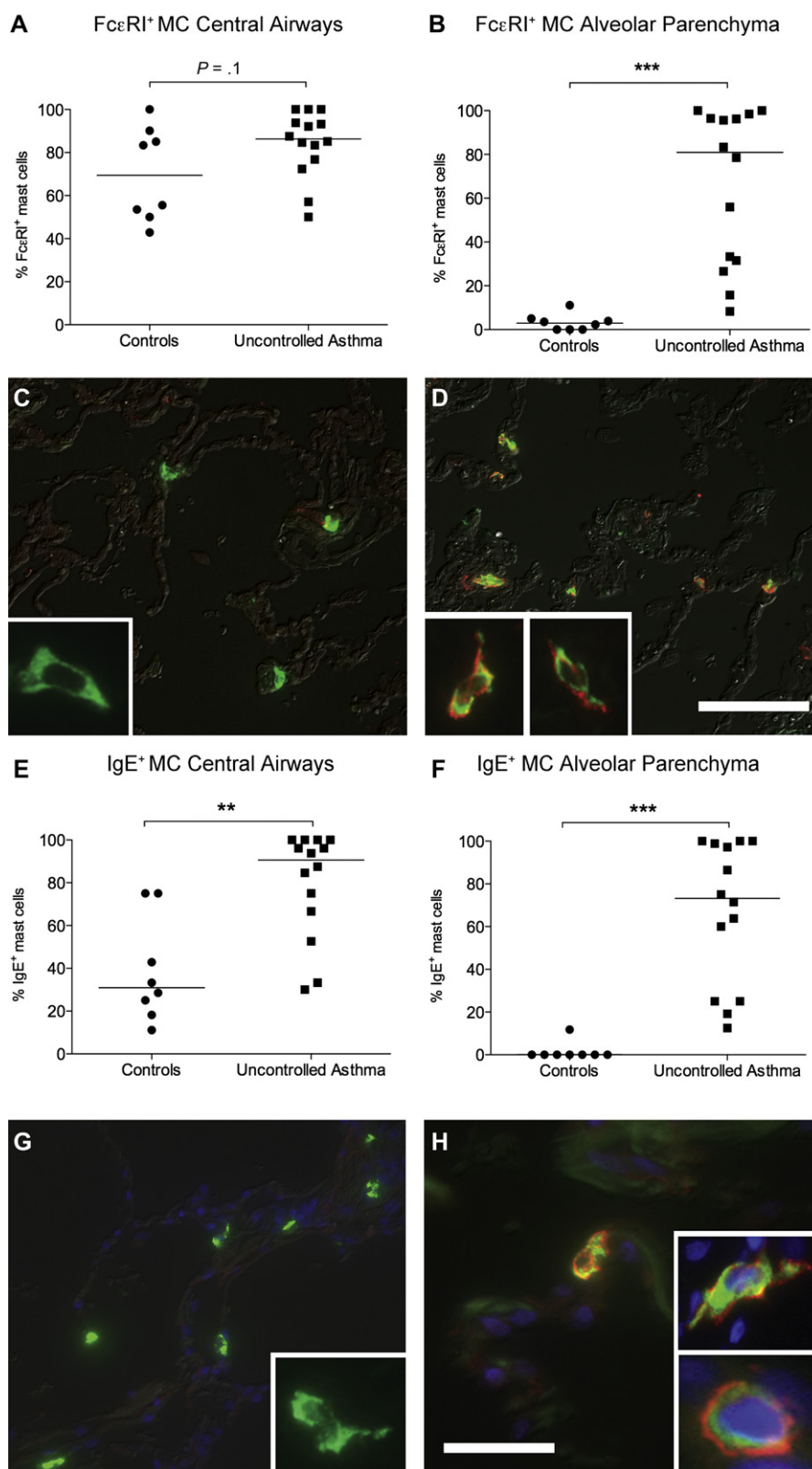


FIG 2. A and B, Mast cell (MC) expression (%) of FcεRI. Representative micrographs of FcεRI⁺ mast cells in alveolar parenchyma from controls (C) and patients with asthma (D). **E and F,** mast cell-bound IgE (%). Representative micrographs of IgE⁺ mast cells in alveolar parenchyma from controls (G) and patients with asthma (H). Scale bar, C, D, G = 50 μm; H = 25 μm. Insets represent image ($\times 600$) of mast cells double-positive for tryptase and FcεRIα or IgE. Horizontal bars indicate median value. Significant differences are shown as $**P < .01$, and $***P < .001$.

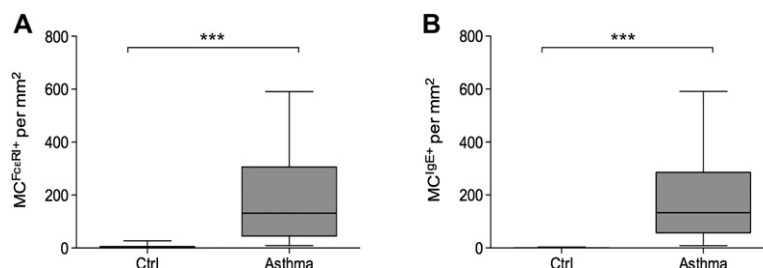


FIG 3. Density of mast cells expressing FcεRIα (**A**) and mast cell-bound IgE (**B**) in the alveolar parenchyma in uncontrolled asthma compared with healthy controls (*Ctrl*). Data are presented as boxes and whiskers.

exposure. Although we could not find increased FcεRI expression in the central airways, previous studies have reported increased numbers of bronchial FcεRI⁺ mast cells in atopic subjects without asthma.³⁸ The vast majority of patients with asthma have rhinitis,⁴⁰ which, especially at severe stages, is a major risk factor for developing asthma.^{41–43} Hence, in future studies it seems important to explore the FcεRI expression on alveolar mast cells in high-risk patients with rhinitis and newly diagnosed patients with asthma and rhinitis. Whether high FcεRI and IgE-expressing alveolar mast cells are present in additional asthma cohorts needs to be investigated further. We could not detect similar changes in other airway diseases characterized by extensive alveolar inflammation and/or remodeling like COPD and CF, despite a rich occurrence of alveolar mast cells in these diseases. However, it should be noted that CF and COPD differ from the uncontrolled asthmatic group, not only in pathological features but also in treatment. Despite their medication, these patients have a significant remaining inflammatory response in the alveolar parenchyma. In this inflammation, the alveolar mast cells have a low expression of the FcεRI.

Although most of the inhaled allergens are deposited in the conducting airways, common allergens may well be transported by respirable particles all the way to the alveolar region.^{44–46} For patients sensitized to systemic allergens, the presence of FcεRI⁺ mast cells in the alveolar parenchyma could theoretically contribute to the increased risk of anaphylaxis associated with asthma.⁴⁷ If occurring, an IgE-driven allergic inflammation in the alveolar parenchyma is likely to have pathophysiological implications. Several mast cell mediators have profibrotic and matrix-modulating properties and may thus contribute to the structural alterations that recently have been observed in alveolar tissues from patients with asthma.²⁷

In 2002, Brightling et al¹⁷ showed that the density of mast cells in the airway smooth muscle layer increased in subjects with asthma. This phenomenon was also found in the current study, in which an increased density of MC_{TC} cells, but not MC_T cells, was found in the bronchial smooth muscle layer of patients with uncontrolled asthma compared with the same compartment in healthy controls. Given that this mast cell subtype is thought to be steroid insensitive, and that several mast cell mediators have the ability to cause airway bronchoconstriction, hyperresponsiveness, and remodeling, this finding supports the proposed pathophysiological effects of smooth muscle-associated mast cells in asthma.^{48,49}

In this study, we found no direct correlations between mast cell parameters and lung function values within the uncontrolled asthma cohort (see Table E4). However, the number of patients was small, and previous studies have shown that conventional lung functional parameters might not accurately represent distal

lung inflammation.⁵⁰ Indeed, it has been demonstrated that measurements of thoracic gas volume and total lung capacity better represent peripheral inflammation, and that these parameters correlate to, for example, distal eosinophilic inflammation in patients with nocturnal asthma.³⁷

In support of a beneficial effect of the ongoing treatment with ICS, the MC_T population decreased in central airways of patients with asthma, whereas the MC_{TC} numbers increased. Because steroids have previously been shown to reduce mast cell numbers in central airways and to affect mainly the MC_T population,⁵¹ this observation implies an effect of ICS on mast cells in the bronchi. In contrast, both MC_T and MC_{TC} subpopulations increased significantly in the less steroid-exposed alveolar parenchyma, which indicates that alveolar mast cell populations are not well targeted by conventional ICS therapy.

Our data support the notion that patients who do not respond to conventional ICS therapy may have a peripheral airway inflammation and should thus benefit from treatment strategies with improved targeting of the distal lung.^{11,52} ICSs, which are the foundation treatment of choice for patients with asthma,^{6,53,54} were originally developed to treat primarily the central airways. Anti-IgE therapy (omalizumab; Xolair) was developed on the basis of the proposed IgE-driven allergic inflammation in the conducting airways.^{55–57} Omalizumab downregulates both free IgE and FcεRI-bearing mast cells in asthmatic bronchi.^{3,58} Future studies are now needed to investigate whether anti-IgE therapy yields similar effects in the alveolar compartment.

In summary, this study has demonstrated that atopic patients with asthma and persistent symptoms despite conventional ICS therapy have increased MC_T and MC_{TC} populations in the alveolar parenchyma. These expanded populations are characterized by markedly elevated expression of FcεRI and surface-bound IgE. Apart from advancing the concept of a distal airway inflammation in asthma, this observation provides important indications regarding how to improve treatment strategies for uncontrolled asthma.

We thank Karin Jansner and Britt-Marie Nilsson for skillful technical assistance with tissue processing and immunohistochemical staining.

Clinical Implications: The current mast cell alterations in the alveolar parenchyma represent a novel feature of asthma that may have clinical implications and support the rationale to target the distal airways in patients with uncontrolled asthma on ICS.

REFERENCES

1. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008;8:183–92.

2. Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001;344:350-62.
3. Holgate S, Casale T, Wenzel S, Bousquet J, Deniz Y, Reisner C. The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. *J Allergy Clin Immunol* 2005;115:459-65.
4. Holt PG, Macaubas C, Stumbles PA, Sly PD. The role of allergy in the development of asthma. *Nature* 1999;402:B12-7.
5. Holgate ST. Novel targets of therapy in asthma. *Curr Opin Pulm Med* 2009;15:63-71.
6. Global Initiative for Asthma. GINA report, global strategy for asthma management and prevention. 2006. Available at: <http://www.ginasthma.com>. Accessed March 21, 2010.
7. Cazzoletti L, Marcon A, Janson C, Corsico A, Jarvis D, Pin I, et al. Asthma control in Europe: a real-world evaluation based on an international population-based study. *J Allergy Clin Immunol* 2007;120:1360-7.
8. Barnes PJ, Adcock IM. Glucocorticoid resistance in inflammatory diseases. *Lancet* 2009;373:1905-17.
9. de Magalhaes Simoes S, dos Santos MA, da Silva Oliveira M, Fontes ES, Ferneziian S, Garippo AL, et al. Inflammatory cell mapping of the respiratory tract in fatal asthma. *Clin Exp Allergy* 2005;35:602-11.
10. Elliot JG, Abramson MJ, Drummer OH, Walters EH, James AL. Time to death and mast cell degranulation in fatal asthma. *Respirology* 2009;14:808-13.
11. Martin RJ. Therapeutic significance of distal airway inflammation in asthma. *J Allergy Clin Immunol* 2002;109:S447-60.
12. Balzar S, Strand M, Rhodes D, Wenzel SE. IgE expression pattern in lung: relation to systemic IgE and asthma phenotypes. *J Allergy Clin Immunol* 2007;119:855-62.
13. Bergeron C, Hauber HP, Gotfried M, Newman K, Dhand R, Servi RJ, et al. Evidence of remodeling in peripheral airways of patients with mild to moderate asthma: effect of hydrofluoroalkane-flunisolide. *J Allergy Clin Immunol* 2005;116:983-9.
14. Kraft M, Djukanovic R, Wilson S, Holgate ST, Martin RJ. Alveolar tissue inflammation in asthma. *Am J Respir Crit Care Med* 1996;154:1505-10.
15. Bradding P, Walls AF, Holgate ST. The role of the mast cell in the pathophysiology of asthma. *J Allergy Clin Immunol* 2006;117:1277-84.
16. Craig SS, DeBlois G, Schwartz LB. Mast cells in human keloid, small intestine, and lung by an immunoperoxidase technique using a murine monoclonal antibody against tryptase. *Am J Pathol* 1986;124:427-35.
17. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;346:1699-705.
18. Weidner N, Austen KF. Heterogeneity of mast cells at multiple body sites. Fluorescent determination of avidin binding and immunofluorescent determination of chymase, tryptase, and carboxypeptidase content. *Pathol Res Pract* 1993;189:156-62.
19. Andersson CK, Mori M, Bjermer L, Lofdahl CG, Erjefalt JS. Novel site-specific mast cell subpopulations in the human lung. *Thorax* 2009;64:297-305.
20. Andersson CK, Mori M, Bjermer L, Lofdahl CG, Erjefalt JS. Alterations in lung mast cell populations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;181:206-17.
21. Brown JL, Behndig AF, Sekerel BE, Pourazar J, Blomberg A, Kelly FJ, et al. Lower airways inflammation in allergic rhinitis: a comparison with asthmatics and normal controls. *Clin Exp Allergy* 2007;37:688-95.
22. den Otter I, Silva LF, Carvalho AL, Pires-Neto RC, Annoni R, Ferreira DS, et al. High-affinity immunoglobulin E receptor expression is increased in large and small airways in fatal asthma. *Clin Exp Allergy* 2010;40:1473-81.
23. Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol* 2008;8:205-17.
24. Smurthwaite L, Walker SN, Wilson DR, Birch DS, Merrett TG, Durham SR, et al. Persistent IgE synthesis in the nasal mucosa of hay fever patients. *Eur J Immunol* 2001;31:3422-31.
25. Schatz M, Sorkness CA, Li JT, Marcus P, Murray JJ, Nathan RA, et al. Asthma Control Test: reliability, validity, and responsiveness in patients not previously followed by asthma specialists. *J Allergy Clin Immunol* 2006;117:549-56.
26. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008;63(suppl 86):8-160.
27. Nihlberg K, Andersson-Sjoland A, Tufvesson E, Erjefalt JS, Bjermer L, Westergren-Thorsson G. Altered matrix production in the distal airways of individuals with asthma. *Thorax* 2010;65:670-6.
28. Irani AA, Schechter NM, Craig SS, DeBlois G, Schwartz LB. Two types of human mast cells that have distinct neutral protease compositions. *Proc Natl Acad Sci U S A* 1986;83:4464-8.
29. Irani AM, Nilsson G, Miettinen U, Craig SS, Ashman LK, Ishizaka T, et al. Recombinant human stem cell factor stimulates differentiation of mast cells from dispersed human fetal liver cells. *Blood* 1992;80:3009-21.
30. Kleinjan A, McEuen AR, Dijkstra MD, Buckley MG, Walls AF, Fokkens WJ. Basophil and eosinophil accumulation and mast cell degranulation in the nasal mucosa of patients with hay fever after local allergen provocation. *J Allergy Clin Immunol* 2000;106:677-86.
31. Weidner N, Austen KF. Ultrastructural and immunohistochemical characterization of normal mast cells at multiple body sites. *J Invest Dermatol* 1991;96:26S-30S, discussion 30S-31S, 60S-65S.
32. Mori A, Zhai YL, Toki T, Nikaido T, Fujii S. Distribution and heterogeneity of mast cells in the human uterus. *Hum Reprod* 1997;12:368-72.
33. Brown JK, Pemberton AD, Wright SH, Miller HR. Primary antibody-Fab fragment complexes: a flexible alternative to traditional direct and indirect immunolabeling techniques. *J Histochem Cytochem* 2004;52:1219-30.
34. Toivonen R, Mayranpaa MI, Kovanen PT, Savontaus M. Dilated cardiomyopathy alters the expression patterns of CAR and other adenoviral receptors in human heart. *Histochem Cell Biol* 2010;133:349-57.
35. Balzar S, Chu HW, Strand M, Wenzel S. Relationship of small airway chymase-positive mast cells and lung function in severe asthma. *Am J Respir Crit Care Med* 2005;171:431-9.
36. Balzar S, Wenzel SE, Chu HW. Transbronchial biopsy as a tool to evaluate small airways in asthma. *Eur Respir J* 2002;20:254-9.
37. Sutherland ER, Martin RJ, Bowler RP, Zhang Y, Rex MD, Kraft M. Physiologic correlates of distal lung inflammation in asthma. *J Allergy Clin Immunol* 2004;113:1046-50.
38. Humbert M, Grant JA, Taborda-Barata L, Durham SR, Pfister R, Menz G, et al. High-affinity IgE receptor (FcεpsilonRI)-bearing cells in bronchial biopsies from atopic and nonatopic asthma. *Am J Respir Crit Care Med* 1996;153:1931-7.
39. Gelfand EW, Kraft M. The importance and features of the distal airways in children and adults. *J Allergy Clin Immunol* 2009;124:S84-7.
40. Greisner WA 3rd, Settiple RJ, Settiple GA. Co-existence of asthma and allergic rhinitis: a 23-year follow-up study of college students. *Allergy Asthma Proc* 1998;19:185-8.
41. Guerra S, Sherrill DL, Martinez FD, Barbee RA. Rhinitis as an independent risk factor for adult-onset asthma. *J Allergy Clin Immunol* 2002;109:419-25.
42. Linneberg A, Henrik Nielsen N, Frolund L, Madsen F, Dirksen A, Jorgensen T. The link between allergic rhinitis and allergic asthma: a prospective population-based study. *The Copenhagen Allergy Study. Allergy* 2002;57:1048-52.
43. Settiple RJ, Hagy GW, Settiple GA. Long-term risk factors for developing asthma and allergic rhinitis: a 23-year follow-up study of college students. *Allergy Proc* 1994;15:21-5.
44. Custovic A, Woodcock H, Craven M, Hassall R, Hadley E, Simpson A, et al. Dust mite allergens are carried on not only large particles. *Pediatr Allergy Immunol* 1999;10:258-60.
45. Taylor PE, Flagan RC, Miguel AG, Valenta R, Glovsky MM. Birch pollen rupture and the release of aerosols of respirable allergens. *Clin Exp Allergy* 2004;34:1591-6.
46. Taylor PE, Flagan RC, Valenta R, Glovsky MM. Release of allergens as respirable aerosols: a link between grass pollen and asthma. *J Allergy Clin Immunol* 2002;109:51-6.
47. Gonzalez-Perez A, Aponte Z, Vidaurre CF, Rodriguez LA. Anaphylaxis epidemiology in patients with and patients without asthma: a United Kingdom database review. *J Allergy Clin Immunol* 2010;125:1098-104, e1.
48. Berger P, Laurent F, Begueret H, Perot V, Rouiller R, Raherison C, et al. Structure and function of small airways in smokers: relationship between air trapping at CT and airway inflammation. *Radiology* 2003;228:85-94.
49. Berger P, Girodet PO, Begueret H, Ousova O, Perng DW, Marthan R, et al. Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. *FASEB J* 2003;17:2139-41.
50. Wagner EM, Liu MC, Weinmann GG, Permutt S, Bleecker ER. Peripheral lung resistance in normal and asthmatic subjects. *Am Rev Respir Dis* 1990;141:584-8.
51. Bentley AM, Hamid Q, Robinson DS, Schotman E, Meng Q, Assoufi B, et al. Prednisolone treatment in asthma: reduction in the numbers of eosinophils, T cells, tryptase-only positive mast cells, and modulation of IL-4, IL-5, and interferon-gamma cytokine gene expression within the bronchial mucosa. *Am J Respir Crit Care Med* 1996;153:551-6.
52. Hamid Q, Tulic MK. New insights into the pathophysiology of the small airways in asthma. *Ann Thorac Med* 2007;2:28-33.
53. FitzGerald JM, Shahidi N. Achieving asthma control in patients with moderate disease. *J Allergy Clin Immunol* 2010;125:307-11.
54. Barnes PJ. Inhaled glucocorticoids for asthma. *N Engl J Med* 1995;332:868-75.
55. Holgate S, Smith N, Massanari M, Jimenez P. Effects of omalizumab on markers of inflammation in patients with allergic asthma. *Allergy* 2009;64:1728-36.
56. Busse WW. Anti-immunoglobulin E (omalizumab) therapy in allergic asthma. *Am J Respir Crit Care Med* 2001;164:S12-7.
57. Strunk RC, Bloomberg GR. Omalizumab for asthma. *N Engl J Med* 2006;354:2689-95.
58. Bousquet J, Cabrera P, Berkman N, Buhl R, Holgate S, Wenzel S, et al. The effect of treatment with omalizumab, an anti-IgE antibody, on asthma exacerbations and emergency medical visits in patients with severe persistent asthma. *Allergy* 2005;60:302-8.

METHODS

Subjects

COPD and CF cohorts. For comparison with nonallergic inflammatory lung diseases, the current study involved 10 patients with end-stage COPD^{E1} and 5 patients with end-stage CF. The patients with COPD were diagnosed on the basis of the global initiative for chronic obstructive lung disease (GOLD) classifications,^{E2} and patients with CF were diagnosed on the basis of clinical manifestations from the lung and gastrointestinal tract and a positive sweat test.^{E3,E4} Tissue was obtained through lung transplantations. Eight never-smoking subjects were used as controls,^{E5} and lung tissue was obtained in association with lung lobectomy because of suspected lung cancer from otherwise healthy nonatopic individuals.^{E6-E9} All subjects were nonatopic on the basis of a standardized SPT. For patient characteristics and medication details, see Table E1. In addition to the medication presented in Table E1, patients with CF were treated with medication against gastritis and insulin for diabetes mellitus. Some patients also had treatment with pancrelipase, litholytics, diuretics, painkillers, anxiolytics, and sedatives. The patients with COPD (GOLD IV) were in addition treated with vitamins, painkillers, diuretics, anxiolytics, sedatives, antidepressant drugs, and medication against cardiovascular disease (arrhythmia, hypertension, and angina pectoris). All subjects gave their written informed consent to participate in the studies, which were approved by the ethics committee in Lund, Sweden.

Spirometry and methacholine inhalation challenge test

For measurements of lung function, a MasterScope spirometer (v. 4.5; Erich Jaeger GmbH, Würzburg, Germany) was used, and reference values were obtained from Crapo et al.^{E10} Two values of FEV₁ with less than 4% variation were measured, and the better value was recorded as the baseline. If the baseline value was less than 70% of predicted, the methacholine inhalation challenge test was aborted, and patients were excluded from the study. The presence of bronchial hyperresponsiveness was measured with a methacholine inhalation challenge test, which was performed with tidal volume-triggered equipment (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. If the FEV₁ declined more than 20% during the test, the challenge was aborted. A positive test was defined as the cumulative dose that caused a decline in FEV₁ by 20% or more from baseline. When FEV₁ fell below 80% of the baseline value or when the total amount of 2000 µg methacholine was delivered, 400 µg salbutamol were given to the subject. After 10 minutes, a new flow-volume spirometry was carried out to ensure that the subjects were recovering accurately.

Bronchoscopy and collection of bronchial and transbronchial biopsies from patients with uncontrolled asthma, AR, and healthy controls

All subjects were without upper respiratory tract infections within 3 weeks before, or during, the study period. From each patient, central airway biopsy specimens (n = 5 per patient) were taken from the segmental or subsegmental carina in the right lower and upper lobes, followed by sampling of transbronchial biopsy (n = 5 per patient) in the right lower lobe. In total, 300 biopsies were collected. Oxygen was given as needed during and after the procedure. Bronchoscopy was performed after local anesthesia with a flexible bronchoscope (IT160; Olympus, Tokyo, Japan), and transbronchial biopsy specimens were taken with biopsy forceps (FB211D; Olympus) under fluoroscopic guidance in the peripheral right lower lobe at a distance of >2 cm from the chest wall. Fluoroscopy of the right lung was performed immediately and 2 hours after the procedure to rule out significant bleeding or pneumothorax.

Processing of tissue and immunohistochemistry

Bronchial and transbronchial biopsies. All biopsies from patients with uncontrolled asthma and 4 of 5 bronchial and 4 of 5 transbronchial biopsies from the healthy controls were immediately placed

in 4% buffered formaldehyde (fixation time, 1.5–2 hours at room temperature and 15–19 hours at 4°C), dehydrated, and embedded in paraffin. Serial paraffin sections of 3 µm were generated and stored until histologic examination. Sections from all paraffin blocks were stained with Mayer hematoxylin and these were used to select 2 bronchial and 2 transbronchial biopsies from each patient that had a well preserved morphology and were without any crush or mechanically induced stretch artifacts. The selected high-quality biopsies were used for detailed quantification of mast cell-related parameters. The remaining biopsies from the control patients and the rhinitis cohort were immersed in 1% PLP for 4 hours at 4°C. Specimens were embedded in optimal cutting temperature medium (Tissue-Tek, Miles Laboratories, Elkhart, Ind) and frozen. Serial cryosections from all biopsies were generated and stored until histologic assessments.

Surgical lung resections and transplantation from patients with COPD and CF.

In patients with end-stage CF and in patients with COPD, lung tissue was collected in association with lung transplantation. For both groups, multiple tissue blocks (around 15 mm thick) were immediately immersed in fixative after surgery with the aim to get blocks for paraffin embedding that contained most anatomic regions of the lungs. For COPD (30 large tissue blocks, 3 per patient), CF (20 large tissue blocks, 4 per patient), and matched controls (32 large tissue blocks, 4 per patient), samples with central airways and alveolar parenchyma were selected for analysis, and a large number of sequential sections 3 µm in thickness were generated.

Double immunohistochemical staining of MC_{TC} and MC_T.

Immunohistochemical procedures were identically performed in asthma, AR, COPD, and CF. A double staining protocol was used for simultaneous visualization of MC_{TC} and MC_T cells. Rehydration and heat-induced antigen retrieval were performed in DIVA decloaker (Biocare Medical, Concord, Calif), pH 6, in a pressure cooker (2100 Retriever; Prestige Medical Ltd, Blackburn, United Kingdom). Sections were blocked with dual endogenous enzyme blocks that quench endogenous alkaline phosphatase and peroxidase. Chymase-containing mast cells were detected with an anti-chymase antibody, detected with a horseradish peroxidase-conjugated antimouse secondary antibody and the nonpermeable chromogen 3,3'-diaminobenzidine. After a step with double staining blocking (making the first antibody inert to further staining by chemically destroying the antigenicity), the remaining MC_T subclass was visualized with an antitryptase antibody, detected with an alkaline phosphatase-conjugated antimouse secondary antibody and Permanent Red chromogen (Table E2). The immunostaining of sections from all study groups was performed in an identical manner by an automated immunohistochemistry robot (Autostainer; Dako) with EnVision G2 Doublestain System (K5361; Dako). Sections were stained with Mayer hematoxylin for visualization of background tissue, dehydrated to xylene, and mounted in Pertex (Histolab, Gothenburg, Sweden). The modified double staining used in this study for detection of the 2 mast cell subpopulations (MC_T and MC_{TC}) does not detect the tryptase-negative MC_C population described by Weidner and Austen in 1993.^{E11} Our triple staining using fluorochrome-labeled antibodies and specific broad-spectrum filters would detect chymase-only positive mast cells. Notably, among the numerous individual mast cells examined in this study, not a single one was found to belong to the chymase only (tryptase⁻, chymase⁺) category.

Immunohistochemical identification of FcεRI⁺ and IgE⁺ mast cells.

Triple staining with immunofluorescence was used to visualize both MC_{TC} and MC_T populations simultaneously together with the mast cell expression of the IgE receptor (FcεRI) or IgE bound to the surface of mast cells. After antigen retrieval (DIVA decloaker; Biocare Medical) in a pressure cooker (2100 Retriever; Prestige Medical Ltd), sections were blocked with normal goat serum (Vector Laboratories, Burlingame, Calif). Sections were stained for the molecule of interest by using specific and validated primary antibodies (Table E1). The FcεRI⁺ or IgE⁺ immunoreactivity was visualized with a goat antirabbit biotinylated secondary antibody and Alexa-Fluor 555-conjugated streptavidin (Molecular Probes, Eugene, Ore). The MC_{TC} population was visualized with a mouse antichymase antibody and a goat antimouse IgG₁ secondary antibody conjugated to Alexa-Fluor 647 (Molecular Probes). Next, MC_T cells were stained with antitryptase antibodies that were prelabeled with AlexaF-488 fluorochrome (Zenon IgG

labeling kit; Molecular Probes). Cell nuclei were stained with pan-DNA marker (Hoechts 33342; Sigma, Stockholm, Sweden). All rinse steps were in TRIS-buffered saline buffer. The staining with the polyclonal antibody against FcεRI (Proteintech Group, Chicago, Ill) was further confirmed with a mouse mAb (Lifespan Biosciences, Seattle, Wash; Table E1). The 2 antibodies displayed identical staining patterns in central airways and the alveolar parenchyma in healthy controls and patients with asthma. Immunohistochemical procedures were identically performed in asthma, AR, COPD, and CF.

Antibody specificity controls. All antibodies in the current studies have been used extensively in research and routine pathological examinations of human formalin-fixed, paraffin-embedded material; most antibodies have also been validated and certified for pathology-based diagnosis. The specificity of the anti-FcεRI and anti-IgE antibodies was further supported by an exclusive distribution of FcεRI to mast cells and basophils (Andersson et al, April 2010, unpublished data) and strong correlations between FcεRI-positive cells and the presence of surface-bound IgE. For all immunohistochemical procedures, markers, and tissues, staining was absent in sections using isotype-matched control antibodies (Dako) that were used instead of, and in the same concentration as, the primary antibody. The mouse monoclonal isotype IgG₁ controls are antibodies against *Aspergillus niger* glucose oxidase, an enzyme that is neither present nor inducible in mammalian tissues. The rabbit negative control immunoglobulin fraction was isolated from serum from healthy nonimmunized rabbits.

Tissue analysis

Quantification of density of mast cell subtypes. In tissue double-stained for MC_{TC} and MC_T, all mast cells per biopsy of each subpopulation were quantified manually in blind sections (digitally scanned by using ScanScope FL; Aperio, Vista, Calif) in central airways (bronchial biopsies) and the alveolar parenchyma (transbronchial biopsies). In tissue from patients with COPD, CF, and controls, mast cell numbers were quantified in 2 to 4 central airways (defined as cartilaginous airways with a diameter of >2 mm) and the alveolar parenchyma (4 randomly selected 0.5-mm² regions) from each tissue block. The densities (cells per mm² tissue area) of MC_T and MC_{TC} were calculated in the walls of bronchi and in the alveolar septa. The bronchi and alveolar tissue area was calculated by using an ImageScope (Aperio). The area of interest was delineated through manual cursor tracing in blind sections and the image analysis program calculated the area, excluding air spaces so that only tissue (ie, bronchial wall or the alveolar septa) was measured. The approach of excluding airspaces thus gives a more relevant tissue density. We also calculated the proportion of MC_T and MC_{TC} in subregions of bronchi: epithelium, subepithelial tissue (excluding smooth muscle and glands), smooth muscle tissue, and subepithelial glands.

Quantification of FcεRI⁺ and IgE⁺ mast cells. In triple-stained sections, all MC_{TC} and MC_T were examined at the wavelengths corresponding to each marker (ie, tryptase, green, 488 nm; chymase, deep red, 647 nm; and FcεRI⁺ or IgE⁺, red, 555 nm). On the basis of this analysis, each MC_{TC} and MC_T was classified as either positive or negative for FcεRIα (or IgE). The distinction between positive and negative cells was facilitated by the fact that for the vast majority of the cells, the immunoreactivity was either absent or nearly absent (criteria for negative) or clear and intense (criteria for positive). In the patients with uncontrolled asthma and the corresponding healthy controls, all individual mast cells were analyzed in each biopsy. In tissue from the other patient groups, mast cell numbers were quantified in 2 to 4 central airways and the alveolar parenchyma (4 randomly selected 0.5-mm² regions) from each tissue block. By dividing the number of tryptase and chymase-positive cells that were co-positive for FcεRI and IgE, respectively, the proportion (%) of total number of mast cells, and each subtype, positive for FcεRI and IgE were calculated. Sections were analyzed by using the NIS-elements AR 3.0 system (Nikon, Tokyo, Japan), a Nikon Eclipse 80i microscope, and a Nikon DS-Qi1Mc camera.

To strengthen the analysis of FcεRI further, an automated image analysis approach was used to calculate the area of FcεRIα positivity per mast cell in transbronchial biopsies in uncontrolled asthma and healthy controls. Multiple high-resolution images of the whole biopsy were obtained by using a double-band filter for visualization of fluorochrome-labeled antibodies at 488 nm and 555 nm. By using narrow and fixed thresholds for both FcεRIα and tryptase to

exclude any autofluorescence, the percentage of FcεRI positive area (intense pixels) per mast cell was calculated with Visiomorph (Visiopharm, Hørsholm, Denmark).

Statistical analysis

Data were analyzed statistically by using the Mann-Whitney rank-sum test for comparison between 2 groups with GraphPad Prism v. 5 (GraphPad Software Inc, La Jolla, Calif). Spearman rank (r_s) correlation tests were used to investigate significant associations between mast cell densities and mast cell expression of FcεRI and IgE⁺ and lung function. For all outcomes, a P value $\leq .05$ was considered significant (* $P \leq .05$; ** $P < .01$; *** $P < .001$).

RESULTS

Expression of FcεRIα and IgE on bronchial and alveolar mast cells

The increased FcεRIα expression in uncontrolled asthma was further confirmed using a computerized image analysis approach that was used to calculate the area of FcεRIα immunoreactivity on individual mast cells. This analysis showed an increase in FcεRIα expression in patients with asthma in central airways (0.4 [0-2.5] % intense FcεRIα1 pixels per mast cell) compared with controls (0.003 [0-0.01] % intense FcεRIα1 pixels per mast cell; $P = .004$). A similar increase was also found in the alveolar parenchyma in patients with asthma (0.1 [0.01-0.8] % intense FcεRIα1 pixels per mast cell) compared with controls (0.0 [0-0.04] % intense FcεRIα1 pixels per mast cell; $P = .001$). In alveolar tissue, no difference in FcεRIα expression was found between MC_T and MC_{TC} subtypes ($P = .7$ and $.8$, respectively).

Mast cell expression of FcεRIα in nonallergic lung diseases: comparison with COPD and CF

To compare FcεRIα expression on mast cells in central airways and alveolar parenchyma in nonallergic lung diseases, 2 groups with patients undergoing lung transplantation for end-stage COPD (GOLD IV) or CF were used and compared with matched controls. The FcεRIα expression was high in central airways in controls (69 [50-100] % MC^{FcεRIα}) compared with the same compartment in COPD (30 [21-55] % MC^{FcεRIα}; $P = .03$) and CF (30 [24-54] % MC^{FcεRIα}; $P = .008$). In the alveolar parenchyma, the mast cell FcεRI expression was low in controls (0 [0-15] % MC^{FcεRIα}) as well as in COPD (0 [0-6] % MC^{FcεRIα}; $P = .2$) and CF (2 [0-6] % MC^{FcεRIα}; $P = .9$).

Distribution of mast cell subtypes in central airways of patients with uncontrolled asthma compared with healthy controls

The densities (per mm²) of MC_T and MC_{TC} were calculated in the central airway wall (epithelium, subepithelial lamina propria tissue, smooth muscle, and subepithelial glands). Epithelium, subepithelial tissue, and smooth muscle were found in all subjects. Subepithelial glands were detected in 4 of the healthy controls and 8 patients with asthma. No difference in the distribution of mast cells was found for the MC_T subclass in patients with asthma compared with controls (Table E3). However, the MC_{TC} subtype increased in the smooth muscle layer in patients with asthma compared with controls (Table E3).

Correlation analysis between mast cell parameters and patient lung function

In patients with uncontrolled asthma, correlation analysis was performed between mast cell densities, mast cell expression of

FcεRIα, mast cell-bound IgE, and patient characteristics. Statistically significant correlations were found between the density of MC_{TC}/mm^2 and $\% MC^{IgE+}$ in both central airways ($r_s = 0.77$; $P = .005$) and the alveolar parenchyma ($r_s = 0.89$; $P = .009$). The mast cell expression of FcεRIα correlated significantly with the total density of mast cells/ mm^2 ($r_s = 0.81$; $P = .007$) and the density of MC_T/mm^2 ($r_s = 0.71$; $P = .02$). A correlation between the percentage of $MC^{FcεRIα+}$ and MC^{IgE+} was found in the alveolar parenchyma of patients with uncontrolled asthma ($r_s = 0.92$; $P = .007$). No significant correlations were found between mast cell parameters and patient characteristics. However, when including the healthy control group in the analysis, the density of MC_T/mm^2 as well as the proportion of MC_{TC} (%) in central airways correlated with FEV_1 % predicted (Table E4).

REFERENCES

- E1. Andersson CK, Mori M, Bjermer L, Lofdahl CG, Erjefalt JS. Alterations in lung mast cell populations in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010;181:206-17.
- E2. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163:1256-76.
- E3. De Boeck K, Wilschanski M, Castellani C, Taylor C, Cuppens H, Dodge J, et al. Cystic fibrosis: terminology and diagnostic algorithms. *Thorax* 2006;61:627-35.
- E4. Farrell PM, Rosenstein BJ, White TB, Accurso FJ, Castellani C, Cutting GR, et al. Guidelines for diagnosis of cystic fibrosis in newborns through older adults: Cystic Fibrosis Foundation consensus report. *J Pediatr* 2008;153:S4-14.
- E5. Andersson CK, Mori M, Bjermer L, Lofdahl CG, Erjefalt JS. Novel site-specific mast cell subpopulations in the human lung. *Thorax* 2009;64:297-305.
- E6. Baraldo S, Turato G, Badin C, Bazzan E, Beghe B, Zuin R, et al. Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax* 2004;59:308-12.
- E7. Finkelstein R, Fraser RS, Ghezzi H, Cosio MG. Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med* 1995;152:1666-72.
- E8. Grashoff WF, Sont JK, Sterk PJ, Hiemstra PS, de Boer WI, Stolk J, et al. Chronic obstructive pulmonary disease: role of bronchiolar mast cells and macrophages. *Am J Pathol* 1997;151:1785-90.
- E9. Saetta M, Mariani M, Panina-Bordignon P, Turato G, Buonsanti C, Baraldo S, et al. Increased expression of the chemokine receptor CXCR3 and its ligand CXCL10 in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002;165:1404-9.
- E10. Crapo RO, Morris AH, Gardner RM. Reference spirometric values using techniques and equipment that meet ATS recommendations. *Am Rev Respir Dis* 1981;123:659-64.
- E11. Weidner N, Austen KF. Heterogeneity of mast cells at multiple body sites: fluorescent determination of avidin binding and immunofluorescent determination of chymase, tryptase, and carboxypeptidase content. *Pathol Res Pract* 1993;189:156-62.

TABLE E1. Characteristics of patients with CF and COPD and controls

Patient characteristic	Controls (CF and COPD)	CF	COPD (GOLD IV)*
No. of patients	8	5	10‡
Sex (M/F)	2/6	2/3	4/6
Age† (y)	63 (33-76)	30 (23-38)	58.5 (53-66)
Inhaled GCS (y)	0	5	9
Oral GCS (y)	0	3	1
β ₂ -agonist (y)	0	5	9
Anticholinergics (y)	0	1	8
Antibiotics (y)	0	3	0
Mucolytic (y)	0	5	5
Vitamins (y)	0	5	1
Calcium supplement (y)	0	3	7
Lung function			
FEV ₁ % of predicted†	110 (82-141)	31 (22-45)	21.4 (13-27)
FEV ₁ /(F)VC†	86 (66-121)	50 (33-84)	30.2 (17-39)
VC % of predicted†	104 (82-126)	54 (46-70)	ND

F, Female; (F)VC, (forced) vital capacity; GCS, glucocorticosteroids; M, male; ND, not determined; Y, yes.

*Patients have been previously used in explorations of mast cells in COPD. See reference.^{E5}

†Data are given as median (range).

‡n = 10; 1 subject with missing medical history.

TABLE E2. Primary antibodies used for immunohistochemistry

Antibody	Species	Dilution	Clone	Origin	Secondary antibody
Antitryptase	Mouse	1:12,000	G3	Chemicon, Temecula, Calif	EnVision G 2 Double stain System, Dako (Glostrup, Denmark)
Antichymase	Mouse	1:100	CC1	Novocastra, Newcastle upon Tyne, United Kingdom	or direct labeling for triple staining (Zenon; Invitrogen)
Anti-FcεR1α	Rabbit	1:50	(polyclonal)	Proteintech Group, Chicago, Ill	Biotinylated goat antirabbit IgG
Anti-FcεR1α	Mouse	1:300	9E1	Lifespan Bioscience, Seattle, Wash	or
Anti-IgE	Rabbit	1:8,000	(polyclonal)	Dako, Glostrup, Denmark	Biotinylated horse antimouse IgG

Heat-induced antigen retrieval was performed in DIVA decloaker (Biocare Medical, Concord, Calif), pH 6, in a pressure cooker (2100 Retriever; Prestige Medical Ltd, Blackburn, United Kingdom).

TABLE E3. Mast cell distribution in central airways

MC_T density (per mm²)	Controls	Uncontrolled asthma	P value
Epithelium	0.0 (0-6)	0.6 (0-15)	.5
Subepithelial tissue	55.1 (9-146)	15.6 (0-49)	.06
Smooth muscle	3.7 (0-32)	2.5 (0-10)	.6
Subepithelial glands	0.0 (0-0)	0.0 (0-0)	NA
MC_{TC} density (per mm²)			
Epithelium	0.0 (0-1)	0.0 (0-3)	.9
Subepithelial tissue	5.6 (0-11)	10.0 (1-40)	.3
Smooth muscle	1.0 (0-5)	7.1 (0-25)	.01
Subepithelial glands	2.5 (0-13)	4.8 (0-15)	.5

NA, not applicable.

Data presented as median (range). Results significant at $P \leq .05$.

TABLE E4. Correlations between mast cell parameters and patient characteristics in uncontrolled asthma

Parameter 1	Parameter 2	r_s	<i>P</i> value
Central airways			
Density MC _T /mm ²	FEV ₁ % predicted	0.56*	.009
Proportion MC _{TC} (%)	FEV ₁ % predicted	0.54*	.02
MC ^{IgE+} (%)	FEV ₁ % predicted	0.60*	.006
MC ^{IgE+} (%)	PD ₂₀	0.58*	.05
Density MC _{TC} /mm ²	MC ^{IgE+} (%)	0.77	.005
Alveolar parenchyma			
Density MC _{tot} /mm ²	MC ^{FceRIα+} (%)	0.81	.007
Density MC _T /mm ²	MC ^{FceRIα+} (%)	0.71	.02
Density MC _{TC} /mm ²	MC ^{IgE+} (%)	0.89	.009
MC ^{IgE+} (%)	MC ^{FceRIα+} (%)	0.92	.007

Correlation analysis was performed by using the Spearman rank correlation test on patients with uncontrolled asthma (no. of patients, 10). *MC_{tot}*, total number of mast cells; r_s = Spearman rank correlation coefficient; $P \leq .05$ is considered significant.

*Correlation performed on patients with asthma and controls.