Human asthma is characterized by more IRF5+ M1 and CD206+ M2 macrophages and less IL-10+ M2-like macrophages around airways compared with healthy airways

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Human asthma is characterized by more IRF5+ M1 and CD206+ M2 macrophages and less IL-10+ M2-like macrophages around airways compared with healthy airways

To the Editor:

A number of recent reports have shown that lung macrophages are important in maintaining lung homeostasis, yet they can also significantly contribute to the development of asthma.[1,2] This contradiction in functions can be explained by their plasticity.[3] Three main polarization states of macrophages are recognized; 2 types associated with inflammatory responses, called M1 and M2, and 1 with anti-inflammatory responses, called M2-like. M1 macrophages are associated with T_H1 inflammation and are characterized by interferon regulatory factor 5 (IRF5) expression. M2 macrophages are associated with T_H2 inflammation and are characterized by increased expression of CD206 (mannose receptor). M2-like macrophages are associated with downregulation of inflammation and characterized by production of high levels of IL-10.[4]

Because CD206+ M2 macrophages are induced by the archetypical asthma cytokines IL-4/IL-13, their numbers and function have been studied more extensively in asthma than those of IRF5+ M1 and IL-10+ M2-like macrophages. Previously, we reported higher numbers of CD206+ M2 in lungs of patients with asthma compared with healthy controls, though in a relatively small sample size cohort. Moreover, we showed that higher CD206+ M2 numbers are associated with more severe asthma[5] and that these CD206+ M2 contribute to asthma development in mouse models of allergic lung inflammation.[1] No reports exist of the numerical presence of IRF5+ M1 or IL-10+ M2-like macrophages in human asthma.

To investigate different macrophage polarization states in patients with asthma versus healthy controls and how they relate to lung function, sex, and inhaled corticosteroid (ICS) use, we used bronchial biopsies from 138 well-characterized patients with asthma and 50 matched healthy controls and stained these for total macrophages using CD68 and doublestained them for the 3 polarization states using IRF5, CD206+, or IL-10+ (for details, see this article's Methods section in the Online Repository at www.jacionline.org). Lung function was assessed and biopsy specimens were taken as described previously.[5] The experimental protocols were approved by the Medical Ethical Committee of the UMCG (METc 2007/007, clinicaltrials.gov NCT00848406), and all subjects gave written informed consent. Table I summarizes the clinical characteristics of all subjects.

We found no differences in the total number of macrophages between healthy males and females and males and females with asthma (Fig 1, *A*). Patients with asthma had higher numbers of IRF5+ M1 and CD206+ M2 macrophages in bronchial biopsies than did healthy individuals, with males having more IRF5+ M1 and females more CD206+ M2 macrophages (Fig 1, *B* and *C*; for representative photos of the stainings, see Fig E1 in this article's Online Repository at www.jacionline.org). The reason for this sex difference is unknown, but may relate to different levels of sex hormones males and females are exposed to. Sexually disparate polarization of macrophages has also been shown in a model of myocarditis.[6] In the latter study, macrophage polarization was

TABLE I. Patients' characteristics (medians + ranges)*

Characteristic	Total asthma (n = 138)	Healthy (n = 50)	Statistical difference	Females with asthma	Males with asthma	Statistical difference
Sex: males/females	70/68	29/21	NS	0/68	70/0	NS
Age (y)	49 (17 to 71)	51 (19 to 73)	NS	45 (17 to 71)	49 (19 to 68)	NS
Body mass index (kg/m^2)	26.7 (19 to 44.2)	23.4 (19 to 35.8)	NS	26.5 (20 to 44.2)	26.0 (19 to 35)	NS
Atopy, n (%) (positive skin prick test result)	95 (69)	18 (37)	P = .0001	45 (67)	52 (75)	NS
Current smoking, n (%)	30 (22)	17 (34)	NS	11 (16)	19 (27)	NS
ICS use, yes/no	57/81	0/50	P = .0001	30/38	27/43	NS
β_2 agonist use, yes/no	71/67	ND	–	34/34	36/34	NS
IgE	78 (1 to 1668)	ND	–	64 (4 to 1668)	105 (1 to 1662)	NS
PC_{20}AMP (mg/mL)	198 (0 to 640)	640 (66.7 to 640)	P = .0001	640 (0 to 640)	151 (0.02 to 640)	NS
FEV_1 (% predicted)	93 (34 to 134)	111 (88 to 139)	P = .0001	99 (53 to 134)	88 (34 to 126)	P = .0001
FEV_1/FVC (%)	72 (40-96)	78 (68 to 93)	P = .0001	75 (53 to 96)	69 (40 to 89)	P = .0001
MEF_{50} (% predicted)	73 (17 to 152)	88 (57 to 136)	P = .0011	81 (31 to 152)	65 (17 to 147)	P = .0074
Blood eosinophils ($\times 10^9$/L)	0.17 (0.26 to 0.97)	0.13 (0.02 to 0.36)	P = .0386	0.15 (0.02 to 0.90)	0.19 (0.04 to 0.97)	NS
Blood neutrophils ($\times 10^9$/L)	3.3 (1.4 to 66.7)	2.8 (1.3 to 7.6)	P = .0058	3.3 (1.4 to 66.7)	3.4 (1.4 to 6.08)	NS
Lung eosinophils (numbers per 0.1 mm^2 tissue area)	1.8 (0 to 40)	ND	—	1.4 (0 to 32)	1.9 (0 to 40)	NS
Lung neutrophils (numbers per 0.1 mm^2 tissue area)	6.2 (0 to 46)	ND	—	6.3 (0 to 29)	5.5 (0 to 46)	NS
Lung CD3+ T cells (numbers per 0.1 mm^2 tissue area)	67.6 (4.2 to 294)	ND	—	76.3 (23 to 216)	58.8 (4.2 to 294)	NS
NO alveolar (ppb)	5.3 (0.88 to 51.7)	3.7 (−0.28 to 67.8)	P = .0006	4.8 (0.88 to 18.34)	5.6 (1.93 to 51.7)	P = .045
NO bronchial (nL/s)	0.7 (0.08 to 10.4)	0.6 (−2.67 to 3.6)	NS	0.7 (0.09 to 4.12)	0.7 (0.08 to 10.4)	NS
BM thickness (μm)	5.9 (2.8 to 12.6)	3.1 (1.6 to 7.0)	P = .0001	5.4 (3.1 to 9.6)	6.3 (2.8 to 12.6)	P = .0168
Goblet cells (number per 1 mm BM)	36 (0 to 220)	ND	—	36 (0 to 119)	36 (1.8 to 220)	NS
Collagen III deposition area (%)	28 (3 to 66)	ND	—	28 (5.8 to 59)	28 (3 to 66)	NS

BM, Basement membrane; *MEF_{50}*, maximum expiratory flow rate at 50% of vital capacity; *NA*, not applicable; *ND*, no data; *NS*, not significant; *$PC_{20}AMP$*, provocative concentrations of adenosine 5'monophosphate causing a 20% fall in FEV_1.
*Most patients showed mild to moderate asthma symptoms.

influenced by sex hormone–driven IFN-γ or IL-4 production by natural killer cells: testosterone was found to favor IFN-γ production by natural killer cells, whereas estrogen favored IL-4 production.

These data confirm our findings on CD206+ M2 macrophages in asthma and show, in a bigger cohort, that macrophages are differentially polarized in asthma. Although asthma is dominated by the presence of IL-4 and IL-13 that can induce CD206+ M2 macrophages, previous indirect evidence suggested that IRF5+ M1 macrophages may also be present in asthma.[7] We have now provided direct evidence that IRF5+ M1 macrophages are indeed also higher in asthma. Murine studies suggest that IRF5+ M1 macrophages can aggravate established asthma[8] and this is supported by our results because higher numbers of IRF5+ M1 macrophages in airway wall biopsies were associated with slightly lower FEV_1/forced vital capacity values (Fig 1, *E*).

Because males with asthma had a significantly lower lung function than did females with asthma (Table I), the above reported sex differences in IRF5+ M1 and CD206+ M2 macrophages may be influenced by the difference in lung function. Therefore, a distinction was made between males and females with asthma with lower lung function (FEV_1 <85% predicted) and with better lung function (FEV_1 >85% predicted). Males with asthma with both FEV_1 values of less than 85% and FEV_1 values of more than 85% predicted had higher numbers of IRF5+ M1 macrophages than did similar females with asthma (Fig 1, *G* and *H*), whereas females with both FEV_1 values of less than 85% and FEV_1 values of more than 85% predicted had higher numbers of CD206+ M2 macrophages than did males (Fig 1, *I* and *J*). Therefore, it is unlikely that the differences found between males and females were caused by differences in lung function.

Numbers of IL-10+ M2-like macrophages were not significantly different between sexes, but both male and female patients with asthma had lower numbers of IL-10+ M2-like macrophages than did healthy individuals (Fig 1, *D*). This finding could explain previous *in vitro* data that macrophages from lavage fluid of patients with asthma produce less IL-10 than do macrophages from healthy subjects.[9] Some studies have also reported higher levels of IL-10 in subjects with asthma as compared with healthy subjects. An explanation for these different findings could be the use of ICS by most of the patients with asthma in these latter studies, which may have induced IL-10 production by macrophages.[9] This is in line with our results showing that ICS treatment was accompanied by higher numbers of IL-10+ M2-like macrophages (Fig 1, *N*). In addition, we found that a higher number of IL-10+ M2-like macrophages was associated with a slightly better lung function (Fig 1, *F*). This is of interest because these macrophages may therefore be beneficial in reducing asthma symptoms. In accordance, we found lower numbers of IRF5+ M1 macrophages in patients with asthma who used ICS than in nonusers (Fig 1, *L*), suggesting that these macrophages are susceptible to treatment. CD206+ M2 macrophages did not appear to be susceptible to ICS treatment (Fig 1, *M*). However, IL-10+ M2-like macrophages also express CD206 and after subtraction of the IL-10+ subset, we also found lower numbers of CD206+ macrophages in ICS users, suggesting that this polarization state may also be susceptible to ICS inhibition (data not shown). This highlights a limitation of subdividing macrophages

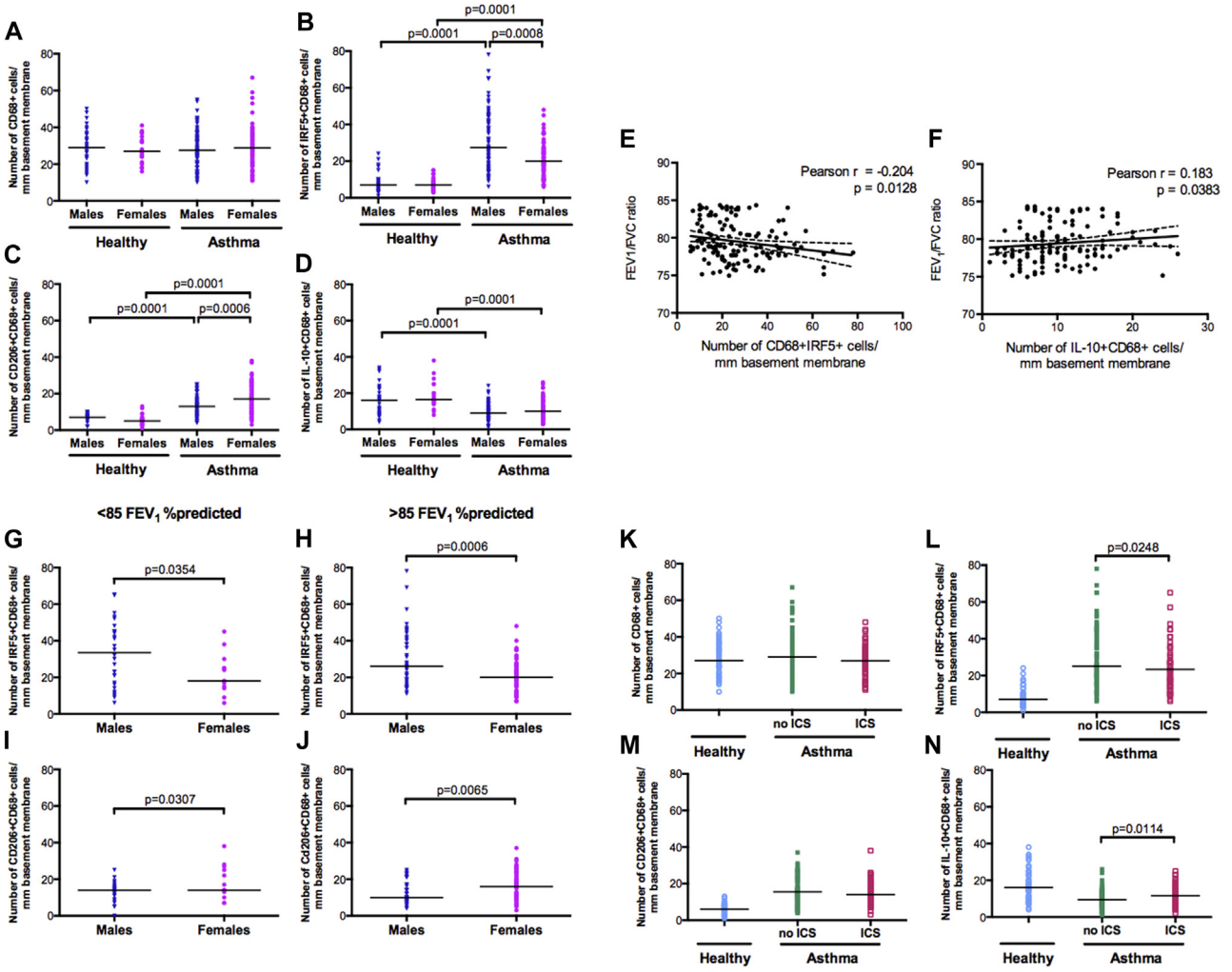

FIG 1. Aligned dot plots with line shown at median of macrophages in bronchial biopsies of healthy subjects and males and females with asthma. Number of all CD68+ cells **(A)**, IRF5+CD68+ **(B)**, CD206+CD68+ **(C)**, and IL-10+CD68+ cells **(D)**. Linear regression of IRF5+CD68+ cells **(E)** and FEV_1/FVC ratio, and IL-10+CD68+ cells **(F)** and FEV_1/FVC ratio in patients with asthma. Number of IRF5+CD68+ cells and CD206+CD68+ cells in male and female patients with asthma with a lower FEV_1 than 85%predicted (**G** and **I**) and with a higher FEV_1 than 85%predicted (**H** and **J**). Number of all CD68+ cells **(K)**, IRF5+CD68+ **(L)**, CD206+CD68+ **(M)**, and IL-10+CD68+ cells **(N)** in healthy subjects and patients with asthma using ICS and not using ICS. *FVC,* Forced vital capacity.

into 3 polarization states while *in vivo* they appear as a continuous spectrum rather than discrete subsets.[3] The total number of CD68+ macrophages is lower than the sum of the 3 polarizations states, indicating that there is indeed overlap in markers. We also checked whether the ratio of CD206+ M2 macrophages to IRF5+ M1 macrophages was in any way correlated to lung function parameters because both these macrophages are found to be higher in asthma, but no significant correlations were found (data not shown).

In conclusion, human asthma is characterized by higher numbers of M1 and M2 macrophages and lower numbers of M2-like macrophages. Strategies to boost numbers of IL-10+ M2-like macrophages may be a good avenue to explore for novel asthma treatments.[E11,E12] For a number of patients with asthma, this can be done through ICS treatment, but for those patients not responding to ICS, novel macrophage-polarizing therapies may be an interesting option because these patients are generally difficult to treat.

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Role of TGF-β in anti-rhinovirus immune responses in asthmatic patients

To the Editor:

The majority of viral infections of the airways are associated with asthma exacerbations in children. Two thirds of these viral infections are caused by rhinovirus, and hospital admissions for asthma correlate with the seasonal peak of rhinovirus infections.[1]

TGF-β is a cytokine known to induce forkhead box $P3^+$ (FoxP3) regulatory T (Treg) cells and retinoic acid–related orphan receptor (ROR) γt^+ T_H17 cells in combination with IL-2 or IL-6, respectively, but also to inhibit the differentiation of T_H1 and T_H2 cells.[2]

Because TGF-β and rhinovirus infection both influence asthma exacerbation and TGF-β also induces rhinovirus replication,[3] in this study we analyzed the effect of rhinovirus infection on TGF-β and the role of TGF-β on rhinovirus infection by analyzing asthmatic and nonasthmatic preschool children recruited in the European study Post-infectious Immune Reprogramming and Its Association with Persistence and Chronicity of Respiratory Allergic Diseases (PreDicta) and a murine model of asthma. The clinical data of the analyzed cohorts of children are reported in Table E1 and in the Methods section in this article's Online Repository at www.jacionline.org. In asthmatic children, in 66.6% of the cases, a viral infection was a triggering factor for development of the disease. Rhinovirus was the most common respiratory virus detected in the airways of these children (see Table E2 in this article's Online Repository at www.jacionline.org).

To investigate the role of TGF-β in rhinovirus-induced asthma in children, we analyzed PBMCs from preschool children with and without asthma, which were cultured for 48 hours after 1 hour of *in vitro* exposure to rhinovirus 1B (RV1B) and subjected them to gene array (Fig 1, *A*, and see Tables E3-E5 in this article's Online Repository at www.jacionline.org). Because TGF-β induces Treg cells,[2] we first investigated which genes related to tolerance were significantly regulated by rhinovirus in PBMCs from these children. Here we found that in asthmatic children rhinovirus upregulated immunosuppressive genes, such as cytotoxic T lymphocyte–associated protein 4 *(CTLA4)* and indoleamine 2,3-dioxygenase *(IDO)*, programmed death ligand 1 (PD-L1; *CD274*), and interferon-induced transmembrane protein 2 (*IFITM2*; Fig 1, *B* and *C*). Consistent with the array data, we found that *IDO1* was upregulated in PBMCs of asthmatic children cultured with rhinovirus compared with those of control children (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). This regulation was found to be independent from steroids because dexamethasone significantly downregulated *IDO* in PBMCs (see Fig E1, *B*).

Because TGF-β is secreted in a latent complex consisting of 3 proteins (TGF-β, the inhibitor latency-associated protein [LAP], and the ECM-binding protein LTBP), we also analyzed these and other TGF-β–inhibitory proteins. We noticed that TGF-β–inhibitory genes, such as *TGIF2* and *LAP3*, were upregulated in rhinovirus-treated PBMCs from asthmatic children. Moreover, rhinovirus inhibited genes that cleave viruses, such as *RNASE1*, in PBMCs from children with asthma (Fig 1, *B* and *C*). By contrast, in control children rhinovirus did not significantly regulate these genes. In these children other factors were found to be significantly regulated by rhinovirus, such as lymphocyte antigen 6E (Fig 1, *D* and *E*), a protein involved in the TGF-β pathway. Moreover, we found that in PBMCs from control children, rhinovirus induced IL-32 (Fig 1, *C* and *D*). Expression of this protein is known to induce the production of IL-6 and TNF-α and might thereby modulate immune responses.[4]

In subsequent experiments we analyzed in more detail the regulation of TGF-β in a larger group of children in the same cohort. Among PBMC supernatants, TGF-β protein was detected in high amounts in untreated cell-culture supernatants in both asthmatic and control children. However, after *ex vivo* challenge with rhinovirus, TGF-β protein expression was found to be significantly decreased (Fig 2, *A*), although *TGFB* mRNA expression remained constant (Fig 2, *B*). Because rhinovirus infection suppressed TGF-β release, we assumed that rhinovirus facilitates TGF-β binding to the cell membrane, and for this reason, we could not detect it in the supernatants of rhinovirus-infected PBMCs.

To prove this concept of a viral immune escape mechanism, we analyzed the expression of *TGFBRII* in PBMCs in the presence or absence of *in vitro* rhinovirus infection. We found that PBMCs isolated from control and asthmatic children and infected with

METHODS

Subjects

Bronchial wall biopsies of patients with asthma (n = 138) and healthy individuals (n = 50) originated from cohorts previously studied by our group.[E1,E2] The experimental protocols were approved by the Medical Ethical Committee of the UMCG (METc 2007/007, clinicaltrials.gov NCT00848406), and all subjects gave written informed consent. Table I presents the clinical characteristics of all subjects. All lung function parameters were significantly lower in patients with asthma than in healthy individuals. Most patients showed mild to moderate asthma symptoms. Male patients had a significantly lower lung function as assessed by FEV_1 (% predicted) and maximal expiratory flow (% predicted) than did female patients.

Patients with asthma

Patients with asthma had a doctor's diagnosis of asthma according to Global Initiative for Asthma (GINA) guidelines and documented reversibility and bronchial hyperresponsiveness (BHR) to histamine in the past. Patients with asthma were extensively reexamined (for study design, see Broekema et al[E1,E3]) and were included in the study if they showed a positive adenosine 5'monophosphate (AMP) provocation test result (PC_{20}AMP < 320 mg/mL), or if this was more than 320 mg/mL a positive histamine provocation test result (PC_{20}histamine < 32 mg/mL).

Healthy individuals

Healthy individuals were included if they met the following criteria: (1) normal pulmonary health according to the physician, (2) normal spirometry defined as an FEV_1 value of 80% predicted or more, an FEV_1/forced vital capacity greater than the lower limit of normal, (3) no bronchodilator reversibility (defined as an increase in FEV_1 of <10% of the predicted value after the administration of 400 μg salbutamol), and (4) no BHR to methacholine. Never-smokers were defined as subjects who had not smoked during the last year, had never smoked for as long as 1 year, and had not smoked more than 0.5 pack-years.

Lung function

A daily-calibrated spirometer was used to perform lung function tests according to standardized guidelines as previously described.[E4] FEV_1 was measured with a calibrated water-sealed spirometer (Lode Spirograph D53, Lode Instruments, Groningen, The Netherlands) according to standardized guidelines[E4,E5] After administration of 400 μg albuterol, the reversibility of FEV_1 (% predicted) was measured. Provocation tests were performed as published previously.[E3,E6] Subjects received an initial nebulized 0.9% saline challenge, followed by doubling concentrations of AMP (0.04-320 mg/mL) by 2-minute tidal breathing at intervals of 5 minutes. BHR to histamine was measured by doubling concentrations ranging from 0.13 to 32 mg/mL using the 30-second tidal breathing method.[E7]

Collection and processing of bronchial biopsies

Bronchial biopsies were collected with a flexible bronchoscope (type Olympus BF P20 or BF XT20; Olympus, Center Valley, Pa) under local anesthesia. The biopsies were obtained from segmental divisions of the main bronchi and thereafter fixed in 4% formalin. After processing, the biopsies were embedded in paraffin and cut into sections of 3-μm thickness.

HISTOLOGY

To identify macrophage subsets in bronchial biopsies, a general macrophage marker CD68 was used in combination with subset-specific markers. Our previous studies in lungs and livers showed that IRF5 is a selective marker for the presence of proinflammatory macrophages when combined with the general macrophage marker CD68.[E8-E10]

Numbers of M1 macrophages were determined by double staining of CD68 (anti-CD68, DAKO, Heverlee, Belgium) and IRF5 (anti-IRF5, ProteinTech Europe, Manchester, UK), M2 macrophages by double staining of CD68 (anti-CD68, Abnova, Heidelberg, Germany) and CD206 (anti-CD206, Serotec, Puchheim, Germany), and M2-like macrophages by double staining of CD68 (anti-CD68, DAKO) and IL-10 (anti–IL-10, Hycult Biotech, Uden, The Netherlands) using standard immunohistochemical procedures. In short, sections were deparaffinized and antigen retrieval was performed by overnight incubation in Tris-HCL buffer, pH 9.0, at 80°C; thereafter, sections were incubated with rabbit anti-IRF5 followed by mouse anti-CD68 or mouse anti-CD206 followed by rabbit anti-CD68. Next, sections were incubated with the 2 secondary antibodies together: horseradish peroxidase (HRP)-conjugated goat-antimouse antibody and alkaline phosphatase (AP)-conjugated goat-antirabbit antibody.

For IL-10 stainings, antigen retrieval was performed by heating the sections in citrate buffer at pH 6.0 for 10 minutes at subboiling temperature. The sections were pretreated with 1% BSA (Sigma Aldrich, Zwijndrecht, The Netherlands) and 5% milk powder in PBS for 30 minutes and incubated with rabbit anti–IL-10 overnight. Subsequently, the sections were incubated with mouse anti-CD68 followed by the 2 secondary antibodies together: HRP-conjugated goat-anti-rabbit antibody and alkaline AP-conjugated goat-anti-mouse antibody.

The AP-conjugated antibodies were first visualized using an immunoalkaline phosphatase procedure with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium as chromogen (Vector, Burlingame, Calif). Next, the HRP-conjugated antibodies were visualized with ImmPACT NovaRED (Vector, Burlingame, Calif) as chromogen.

All stainings were quantified by morphometric analysis using ImageScope analysis software (Aperio, Vista, Calif). A blinded observer manually counted the double-positive cells in whole bronchial biopsy sections per length of intact basement membrane and extending 100 μm into the intact submucosa, excluding vessel and smooth muscle. Data were expressed as the number of positive cells per millimeter of basement membrane.

Statistics

Normality of distributions was assessed using a D'Agostino-Pearson omnibus test. When data were not normally distributed, log or square root transformations were performed. Student *t* tests were used to compare macrophage subsets between 2 groups. Data are shown as aligned dot plots with line at median. Pearson correlations were performed on macrophage subsets and clinical characteristics. *P* values of less than .05 were considered significant. Data were analyzed using Graphpad Prism 6 (GraphPad Software, La Jolla, Calif).

Initial multiple regression analyses were performed within the asthma population to explain the contribution of each macrophage subset (independent variable of model 1: IRF5+ macrophages, model 2: IL-10+ macrophages, and model 3: CD206+ macrophages) to the variation of lung function and inflammation (dependent variable). All models were adjusted for sex, age, height, and current smoking. *P* values of less than .05 were considered significant. Data were analyzed using IBM SPSS Statistics version 22.

None of the macrophage subsets was associated with age, current smoking (no/yes), eosinophils, neutrophils, IgE levels, or

airway remodeling, as reflected by basement membrane thickness, goblet cell numbers, and collagen III deposition. Therefore, the results of the multiple regression analyses are not shown.

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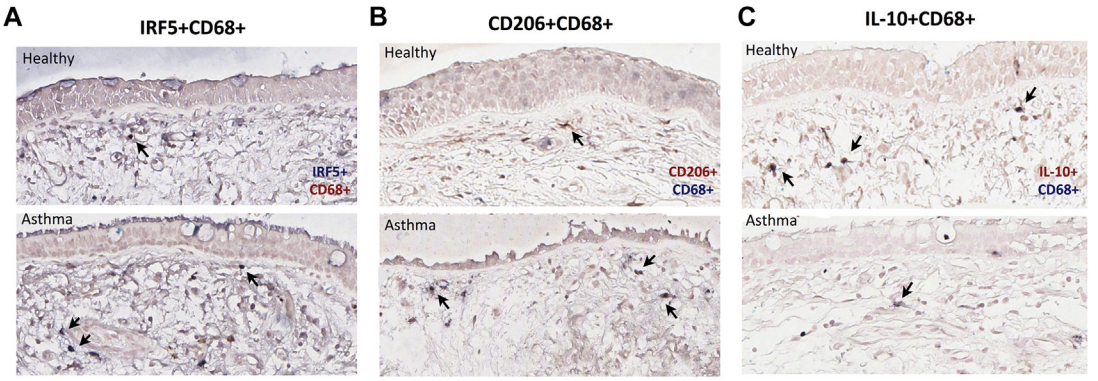

FIG E1. Representative photos of IRF5+CD68+ **(A)**, CD206+CD68+ **(B)**, and IL-10+CD68+ cells **(C)** (magnification 200×).