Phenotypic characterization of lung macrophages in asthmatic patients: Overexpression of CCL17

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Background: Studies with monocyte-derived macrophages (MDMs) and animal models have suggested a role for alternatively activated (M2) macrophages in asthmatic inflammation, but *in vivo* evidence for this phenotype in human asthma is lacking.

Objective: To characterize the phenotype of lung macrophages from asthmatic patients in relation to disease severity and treatment.

Methods: M2 biomarkers were first identified by using MDMs exposed to T_H2 cytokines and then used to phenotype sputum and bronchoalveolar lavage (BAL) macrophages from 12 healthy control subjects, 12 patients with mild asthma, and 14 patients with moderate asthma and to assess the effects of corticosteroids and phosphatidylinositol 3-kinase (PI3K) inhibitors.

Results: Sputum macrophages from asthmatic patients expressed significantly more CCL17 mRNA but less CD163 than macrophages from healthy subjects. However, none of the other M2 biomarkers were differentially expressed in asthmatic patients, and *ex vivo* BAL cells spontaneously produced similar amounts of M2 cytokines/chemokines (IL-10, CCL17, and CCL22). CCL17 mRNA overexpression correlated weakly but significantly with sputum eosinophilia ($P = .0252$) and was also observed in macrophages from patients with moderate asthma treated with inhaled steroids, suggesting relative insensitivity to inhibition by corticosteroids. The PI3K inhibitor LY294002 inhibited basal CCL17 release from BAL cells and IL-4–stimulated release from MDMs.

Conclusions: This study does not support the existence in human asthma of the full M2 phenotype described to date but points to upregulation of CCL17 in both patients with mild and those with moderate asthma, providing a further source for this ligand of $CCR4^+$ cells that contributes to airways inflammation. CCL17 expression is corticosteroid resistant but suppressed by PI3K enzyme inhibitors. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: *Asthma, macrophage, chemokine, steroid*

Asthma is a complex airways inflammatory disease involving several cell types, with most research focusing on eosinophils and $CD4^+$ T_H2 cells,[1,2] the latter being an important source of the cytokines IL-4, IL-5, and IL-13, which are key drivers of responses to allergens.[3] The role of macrophages in driving allergic airways disease has been largely overlooked,[4] even though they are the most prevalent immune cell type in the lungs.

Macrophages have been broadly characterized as either classically activated (M1) or alternatively activated (M2) based on phenotypes observed when macrophages are cultured *in vitro* in the presence of LPS and IFN-γ (M1) or IL-4 or IL-13 (M2).[5] M2 macrophages generally express increased levels of receptors involved in phagocytosis, such as CD206,[6] CD163,[7] and macrophage galactose C-type lectin (CLEC10A/CD301),[8] as well as important T_H2 cell chemokines, including the CCR4 ligands CCL17 and CCL22.[5] Recent studies using animal models[5,9] and human monocytes[10] have suggested a role for M2 macrophages in allergic lung inflammation, but evidence of a similar phenotype being relevant to human asthma has been lacking, and it is recognized that there are differences between human and murine M2 expression profiles.[11]

Given that macrophages are the most numerous inflammatory cell type in the airways, where T_H2 cytokine levels are increased, we postulated that lung macrophages from asthmatic patients are of the M2 phenotype. We also hypothesized that macrophages could be a major source of chemokines that attract $CCR4^+$ cells, which we and others have shown as potentially playing a role in asthma.[12,13] Our previous work has demonstrated that $CCR4^+$ T lymphocytes are a major source of T_H2 cytokines[12] and that their recruitment into the airways of asthmatic patients is controlled by the CCR4 ligands CCL17 and CCL22. However, the source of these chemokines has not been fully elucidated, with airway dendritic cells and epithelial cells being implicated to date.[14-17]

In the current study we first identified a panel of M2 biomarkers using monocyte-derived macrophages (MDMs) cultured in M2-polarizing conditions; these biomarkers were then used to phenotype sputum and bronchoalveolar lavage (BAL) macrophages from patients with mild steroid-naive asthma, patients with moderate steroid-treated asthma, and nonatopic control subjects. In addition to studying M1 (CD14 and TNF) and M2 (*CCL17, CLEC10A*, arginase 1 *(ARG1),* IL-10, IL-13, CD163, CD206, and CLEC10A/CD301) biomarkers at the levels of mRNA, intracellular protein, and cell-surface expression, we also assessed the potential of airway macrophages recovered from BAL fluid to produce a series of cytokines/chemokines

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Abbreviations used

AF647:	Alexa Fluor 647
APC:	Allophycocyanin
APC-Cy7:	Allophycocyanin–cyanine 7
Arg1:	Arginase 1
BAL:	Bronchoalveolar lavage
CLEC10A:	Macrophage galactose C-type lectin (CD301)
COPD:	Chronic obstructive pulmonary disease
FACS:	Fluorescence-activated cell sorting
LDH:	Lactate dehydrogenase
Lin:	Lineage
M1:	Classically activated (macrophages)
M2:	Alternatively activated (macrophages)
MDM:	Monocyte-derived macrophage
MMP:	Matrix metalloproteinase
NF-κB:	Nuclear factor κB
PE:	Phycoerythrin
PE-AF610:	Phycoerythrin–Alexa Fluor 610
PE-Cy7:	Phycoerythrin–cyanine 7
PerCP-Cy5.5:	Peridinin chlorophyll protein–cyanine 5.5
PI3K:	Phosphatidylinositol 3-kinase

that have been implicated in asthma pathogenesis and have been reported as defining the M1 (TNF, IL-12, IL-6, and IL-8) and M2 (CCL17, CCL22, and IL-10) phenotypes.[5] Having found that the frequency of lung macrophages expressing CCL17 is increased in both steroid-naive and steroid-treated asthmatic patients, we studied the effects of the corticosteroid fluticasone propionate and inhibitors of phosphatidylinositol 3-kinase (PI3K) on *ex vivo* CCL17 production by MDMs and BAL fluid macrophages to explore their therapeutic potential.

METHODS

Subjects

Twelve patients with mild atopic asthma taking short-acting β-agonists alone, 14 patients with moderate atopic asthma requiring inhaled corticosteroids for disease control (classified according to the Global Initiative for Asthma criteria, www.ginasthma.org), and 12 healthy nonatopic control subjects were studied (Table I). All subjects were nonsmokers with no respiratory tract infections for 6 weeks before the study. Atopy was assessed by using skin tests to common aeroallergens. The study was approved by the Southampton and South West Hampshire Research Ethics Committee (reference 08/H0504/138).

Benchmarking of M2 biomarkers by using macrophages derived from blood monocytes

Initial experiments were conducted on MDMs derived from circulating monocytes to establish a panel of biomarkers that reflect M2 macrophages for subsequent phenotyping of lung macrophages from asthmatic and control subjects. Monocytes were isolated to greater than 95% purity by means of magnetic cell sorting and anti-CD14 antibodies and differentiated over 12 days in culture in RPMI-1640 (plus 10% FBS) into macrophages by using 2 ng/mL GM-CSF, a protocol shown to produce macrophages with phenotypic and functional characteristics similar to those of lung macrophages.[18,19] The GM-CSF–containing media were then removed and replaced with media containing 10 ng/mL recombinant human IL-4 or IL-13, and culture was continued for a further 24 hours. To check whether the differentiated cells displayed an M2 phenotype, culture supernatants were analyzed by using ELISA for CCL17 release, whereas the resuspended cells were analyzed for a panel of M2 biomarkers (CD206, CD163, CLEC10A/CD301, *Ccl17*, and *Arg*1) by using a combination of flow cytometry and RT-PCR (see the Methods section in this article's Online Repository at www.jacionline.org).

Sampling of lung macrophages

BAL and sputum induction were performed as previously described.[20] Cells were isolated and immediately processed in fluorescence-activated cell sorting (FACS) buffer containing 2 mg/mL human IgG for flow cytometry and RT-PCR, and aliquots were collected for culture.

Analysis of lung macrophage biomarkers

Flow cytometry. Phenotypic characterization of lung macrophages was performed by means of flow cytometry with a 9-color FACSAria cell sorter and FACSDiva Software (version 5.0.3; BD Biosciences, Oxford, United Kingdom). Cells were incubated in reagents from the Live/Dead Violet Viability/Vitality kit (Invitrogen, Paisley, United Kingdom) and the macrophage detection cocktail, consisting of anti-human CD45 phycoerythrin–Alexa Fluor 610 (PE-AF610; Invitrogen), anti-human CD3 phycoerythrin–cyanine 7 (PE-Cy7) and anti-human HLA-DR allophycocyanin–cyanine 7 (APC-Cy7; all BD Biosciences). By using this protocol, $CD45^{+}CD3^{-}HLA\text{-}DR^{+}$ cells were greater than 90% pure macrophages, as shown by using cytologic analysis of sorted cells (see Fig E1 in this article's Online Repository at www.jacionline.org).

Separate aliquots were additionally incubated in a cocktail of antibodies specific for surface biomarkers (anti-CD206 allophycocyanin [APC] and anti-CD14 peridinin chlorophyll protein–cyanine 5.5 [PerCP-Cy5.5] [both BD Biosciences] or anti-CD301 Alexa Fluor 647 [AF647] and anti-CD163 PerCP-Cy5.5 [both Cambridge Bioscience, Cambridge, United Kingdom]) or isotype control antibodies. For intracellular staining, cell aliquots that had been incubated in the macrophage identification cocktail were first washed with FACS buffer and then incubated in Cytofix/Cytoperm (BD Biosciences). These were further washed in 1× Perm/Wash and incubated with anti-human CD68–phycoerythrin (PE), anti-human TNF APC, anti–IL-10 AF647, or isotype control.

RT-PCR. RNA was extracted from FACS-sorted sputum and BAL macrophages by using a Stratagene Microprep Kit (Stratagene, Amsterdam, The Netherlands). Reverse transcription was carried out with a nanoScript Reverse Transcriptase kit (PrimerDesign, Southampton, United Kingdom). PCR amplifications for M2 biomarkers (CCL17, CLEC10A, and ARG1) were performed on a Bio-Rad iCycler (Hemel Hempstead, United Kingdom) with Precision 2X qPCR Mastermix and PerfectProbe primers (PrimerDesign; for full sequences, see the Methods section in this article's Online Repository). Gene expression was normalized to β_2-microglobulin gene expression and quantified by using the $\Delta\Delta C_T$ method.[21]

Analysis of cytokine release by MDMs and BAL fluid cells

MDMs from 5 subjects were cultured in RPMI-1640 (+10% FBS) for 24 hours and stimulated with 10 ng/mL IL-4 in the presence or absence of a range of concentrations of fluticasone propionate or PI3K inhibitors. Similarly, BAL fluid cells (from 13 asthmatic patients) composed of a median of 75% macrophages (for full differential cell counts, see the Methods section in this article's Online Repository) were cultured in AIM V medium (Invitrogen) without additional stimulants or in the presence of 10 μM LY294002. The medium was FBS free so as to avoid skewing of the macrophage phenotype by factors in serum. After 24 hours, supernatants were retained for cytokine and chemokine analysis.

Culture supernatants were analyzed by using a combination of ELISA (R&D Systems, Abingdon, United Kingdom) and Luminex assay (Bio-Rad) for IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α, IFN-γ, CCL17, and CCL22 (for full details, see the Methods section in this article's Online Repository).

Statistics

Statistical analyses were performed by using the Kruskal-Wallis or Friedman ANOVA test with the Dunn Multiple Comparison test, the Mann-Whitney *U* test, or the Wilcoxon signed-rank test, as appropriate (GraphPad Prism version 3; GraphPad Software, San Diego, Calif). Results were considered significant at a *P* value of less than .05.

TABLE I. Baseline characteristics of volunteer healthy control subjects, patients with mild asthma, and patients with moderate asthma

	HC	MA	MO
No.	12	12	14
Sex (male/female)	5/7	6/6	6/8
Age (y)	23.5 (22-31)	24 (21-29.5)	44 (31.5-49)†‖
Atopy	No	Yes	Yes
FEV_1 (% predicted)	110.5 (102.3-120.3)	99.75 (88.6-109.7)	92.10 (80.35-105)†
FEV_1/FVC ratio	0.85 (0.79-0.88)	0.80 (0.72-0.83)	0.78 (0.70-0.85)
Methacholine PC_{20}	Not achieved	2.59 (0.57-5.22)	1.14 (0.36-5.69)
Inhaled corticosteroids	No	No	Yes
ACQ score	ND	0.43 (0.15-0.71)	1.07 (0.43-1.57)‡
Sputum cells (%)			
Macrophages	59.25 (46.78-73.15)	48.8 (33.5-61.9)	48.8 (42.9-76.05)
Neutrophils	34.9 (17.75-44)	34.15 (19.4-54.65)	36.0 (18.28-50.25)
Eosinophils	0.38 (0.0-0.98)	1.38 (0.75-5.5)*	2.5 (0.63-8.15)*
Lymphocytes	0.25 (0.0-0.50)	0.25 (0.0-0.75)	0.25 (0.0-0.5)
Epithelial cells	3.80 (0.75-8.30)	4.65 (1.0-7.75)	2.13 (1.0-9.25)
BAL fluid cells (%)			
Macrophages	86.55 (74.9-90.15)	77.15 (74.55-84.65)	66.25 (52.75-80.75)*
Neutrophils	3.05 (1.63-5.25)	4.65 (2.38-5.15)	8.90 (5.4-16.05)†§
Eosinophils	0.25 (0.0-0.88)	1.75 (1.0-3.38)*	2.40 (0.63-5.38)†
Lymphocytes	1.0 (0.63-1.38)	1.13 (0.70-2.13)	1.0 (0.38-1.38)
Epithelial cells	9.30 (4.15-17.05)	12.15 (5.70-19.88)	17.15 (10.0-26.05)

Data are expressed as median values (interquartile ranges) to 2 decimal places. Data were analyzed by using the Kruskal-Wallis test followed by the Dunn multiple comparisons test. PC_{20} and Asthma Control Questionnaire data were analyzed by using the Mann-Whitney *U* test.
ACQ, Asthma Control Questionnaire; *FVC*, forced vital capacity; *HC*, healthy control subjects; *MA*, patients with mild asthma; *MO*, patients with moderate asthma; *ND*, not determined.
*$P < .01$ versus healthy control subjects.
†$P < .001$ versus healthy control subjects.
‡$P < .05$ versus patients with mild asthma.
§$P < .01$ versus patients with mild asthma.
‖$P < .001$ versus patients with mild asthma.

RESULTS

Assessment of M2 biomarkers using MDMs

Both IL-4 and IL-13 significantly increased *CCL17* and *CLEC10A* gene expression (Fig 1, *A*, and see Fig E2 in this article's Online Repository at www.jacionline.org), with a trend toward increased *ARG1* gene expression (data not shown) induced by IL-13. T_H2 cytokine–treated MDMs (ie, M2 MDMs) also expressed significantly more HLA-DR and CD206 (Fig 1, *B*) and less CD14 and CD163 on their surface (Fig 1, *B*), with no difference in CLEC10A (CD301) or CD36 expression (data not shown). Small amounts of CCL17 were released from unstimulated MDMs (80.3 ± 39 pg/mL), and this was greatly increased by IL-4 (390.3 ± 204.9 pg/mL) and IL-13 (281.4 ± 257.5 pg/mL). These experiments provided a panel of M2 biomarkers previously reported by others.[5,6,8,22]

M2 cell-surface marker expression on sputum and BAL fluid macrophages

The M2 cell-surface biomarkers identified above were applied to sputum and BAL macrophages from asthmatic patients and control subjects and analyzed by using multichromatic flow cytometry. The only surface biomarker that was differentially expressed was CD163 (Fig 2 and Table II). Similar to M2 MDMs, its expression was reduced in asthmatic patients when compared with that seen in healthy subjects on macrophages in sputum ($P = .0346$, Kruskal-Wallis test) but not in BAL fluid ($P = .2893$, Kruskal-Wallis test). Intracellular TNF and IL-10 expression was also not different (Table II), although BAL macrophages appeared to express more TNF than sputum macrophages. Intracellular IL-13 protein was not detected.

Gene expression analysis of lung macrophages

The 90% purity of macrophages obtained by using the above method of selection was considered insufficient for gene expression analysis because any small contamination with other cell types could be amplified by means of PCR. Therefore we added to the macrophage detection protocol the Lineage (Lin) 1 antibody cocktail (BD Biosciences) to exclude dendritic cells from the analysis. Cells sorted in this way ($CD45^+CD3^-Lin^+HLA\text{-}DR^+$, Fig 3) from both sputum and BAL fluid were, on average, 98% pure macrophages, as ascertained by means of histochemical and morphologic analysis of cytospin preparations. Up to 100,000 events were sorted into RNA lysis buffer and stored at −80°C for RNA extraction and RT-PCR.

Sputum macrophages from healthy control subjects expressed little or no CCL17 mRNA (median value, 0; Fig 4), whereas significantly greater expression was observed in both patients with mild asthma (median value, 3.421) and those with moderate (median value, 7.63) asthma. The CCL17 expression correlated weakly ($r^2 = 0.1493$) but significantly ($P = .0252$) with sputum eosinophilia (Fig 4) but not with neutrophil counts ($r^2 = 0.04$). When these analyses were performed on individual asthma groups (mild asthma: $r^2 = 0.279$, $P = .063$; moderate asthma: $r^2 = 0.016$, $P = .7149$) and healthy control subjects ($r^2 = 0.061$, $P = .1456$) separately, none of these correlations were significant nor was there a significant correlation when asthmatic patients

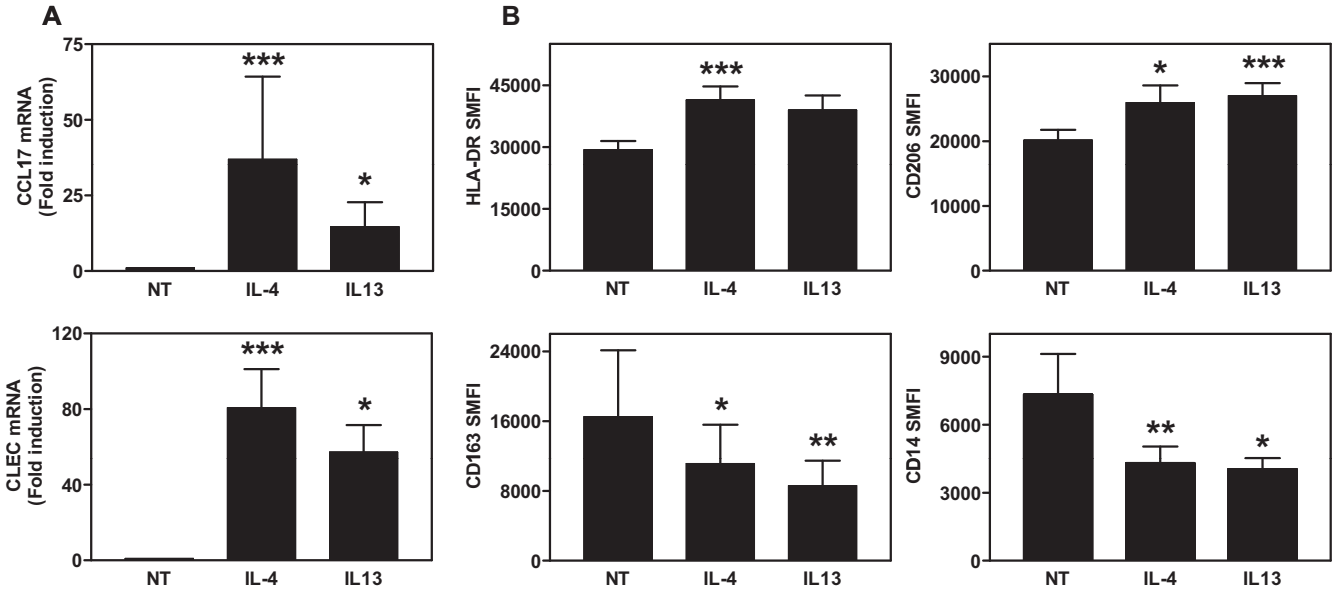

FIG 1. Expression of biomarkers on MDMs stimulated with IL-4 or IL-13. **A,** Gene expression of CCL17 and CLEC10A is expressed as fold induction compared with NT (not treated, mean ± SE, n = 11). **B,** Cell-surface expression data are expressed as specific mean fluorescence intensity (*SMFI*; mean ± SE, n = 12). Data were analyzed by using Friedman ANOVA and the Dunn multiple comparison test compared with NT. $*P < .05$, $**P < .01$, and $***P < .001$.

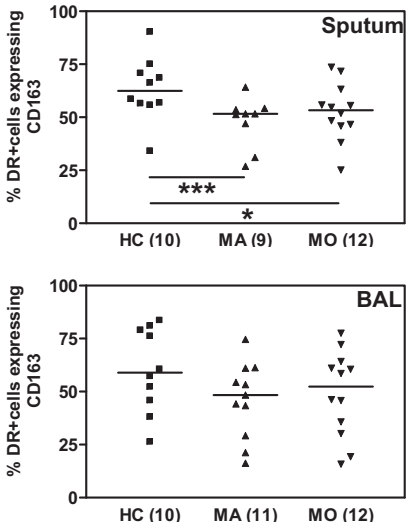

FIG 2. Analysis of lung macrophage M2 marker expression by means of flow cytometry. Expression of cell-surface CD163 on sputum and BAL macrophages of healthy control subjects *(HC)*, patients with mild asthma *(MA)*, and patients with moderate asthma *(MO)* is shown. *Bars* indicate median values, and *numbers in parentheses* indicate *n*. Data are expressed as percentages of Live$^+$CD45$^+$CD3$^-$HLA-DR$^+$ cells and analyzed by using Kruskal-Wallis ANOVA and the Dunn multiple comparison test. $*P < .05$ and $***P < .001$.

were analyzed as a group in the absence of control subjects ($r^2 = 0.109$, $P = .1331$), suggesting the correlation is probably "pulled" into significance because of the inclusion of low-CCL17-expressing, low-eosinophil healthy control subjects. Although a significant increase in CCL17 mRNA expression by BAL cells was observed ($P = .0449$, Kruskal-Wallis test; Fig 4 and see Fig E3 in this article's Online Repository at www.jacionline.org), in contrast to the sputum data, this increase was only significant when comparing patients with mild and moderate asthma by using the Dunn *post hoc* test. There was a trend toward increased expression of CLEC10A mRNA in both sputum and BAL macrophages from patients with mild but not moderate asthma, although this was not statistically significant (data not shown).

Cytokine production by BAL fluid cells

To ascertain whether increased expression of CCL17 mRNA in macrophages translated into increased release of CCL17 protein, freshly isolated BAL fluid cells (containing a median of 75% macrophages) were cultured for 24 hours, and supernatants were analyzed for CCL17 by using the Luminex assay. The lack of observed difference in CCL17 *ex vivo* release by BAL fluid cells (see Fig E4 in this article's Online Repository at www.jacionline.org) suggested no correlation between the *in vivo* gene expression and the *ex vivo* expression of CCL17. Additional cytokine measurements were also performed to assess whether macrophages released a predominance of T_H1 or T_H2 cytokines. None of the T_H2 (IL-4, IL-5, and IL-13) or T_H1 (IFN-γ, IL-2, and IL-12) cytokines were detected, whereas the previously reported M1 (TNF, IL-6, and IL-8) and M2 (IL-10 and CCL22) biomarkers[5] were released at similar levels in all groups (see Fig E4).

Inhibition of CCL17 release by the PI3K inhibitor LY294002 but not corticosteroids

The increased CCL17 mRNA expression observed in lung macrophages from patients with moderate asthma taking inhaled steroids suggested that the production of this chemokine might not be fully responsive to corticosteroids. We therefore investigated whether the *ex vivo* CCL17 release from macrophages could be inhibited by steroid treatment. Initial experiments

TABLE II. Cell-surface and intracellular protein expression by macrophages from healthy control subjects, patients with mild asthma, and patients with moderate asthma

	Sputum			BAL fluid		
	HC	MA	MO	HC	MA	MO
Extracellular						
HLA-DR (SMFI)	59,210 (44,120-75,520)	57,050 (40,780-76,880)	46,280 (42,470-63,470)	49,140 (34,100-56,390)	46,470 (36,650-57,300)	44,470 (30,760-61,870)
CD14 (SMFI)	1,920 (1,498-3,244)	1,729 (1,520-1,993)	1,932 (1,489-2,694)	1,260 (662.5-1,612)	1,328 (717.5-1,808)	1,049 (660-1,922)
CD206 (%)	62.4 (54.7-67.5)	47.7 (28.9-62.5)	56.8 (46.0-76.5)	57.9 (44.1-72.7)	51.6 (42.0-64.4)	57.5 (45.4-70.2)
CD301 (SMFI)	2,785 (1,439-5,921)	2,993 (1,674-3,852)	3,208 (2008-3,909)	2,727 (1,397-5,295)	3,314 (2,917-4,640)	2,540 (1,198-5,318)
Intracellular						
TNF (SMFI)	3,781 (3,174-4,000)	4,464 (3,383-5,366)	3,867 (3379-4,831)	6,675 (5,232-8,380)	6,717 (5,172-8,141)	6,120 (5,473-7,148)
IL-10 (SMFI)	1,296 (1,005-1,735)	1,242 (1,001-1,900)	1,615 (814.5-2,472)	1,324 (722-2,229)	1,976 (1,700-2,179)	1,300 (920-2,014)

HLA-DR, CD14, CD301, TNF, and IL-10 are represented as specific mean fluorescence intensity *(SMFI)*, and CD206 is shown as the percentage of cells staining positively. Data are expressed as median values (interquartile ranges).

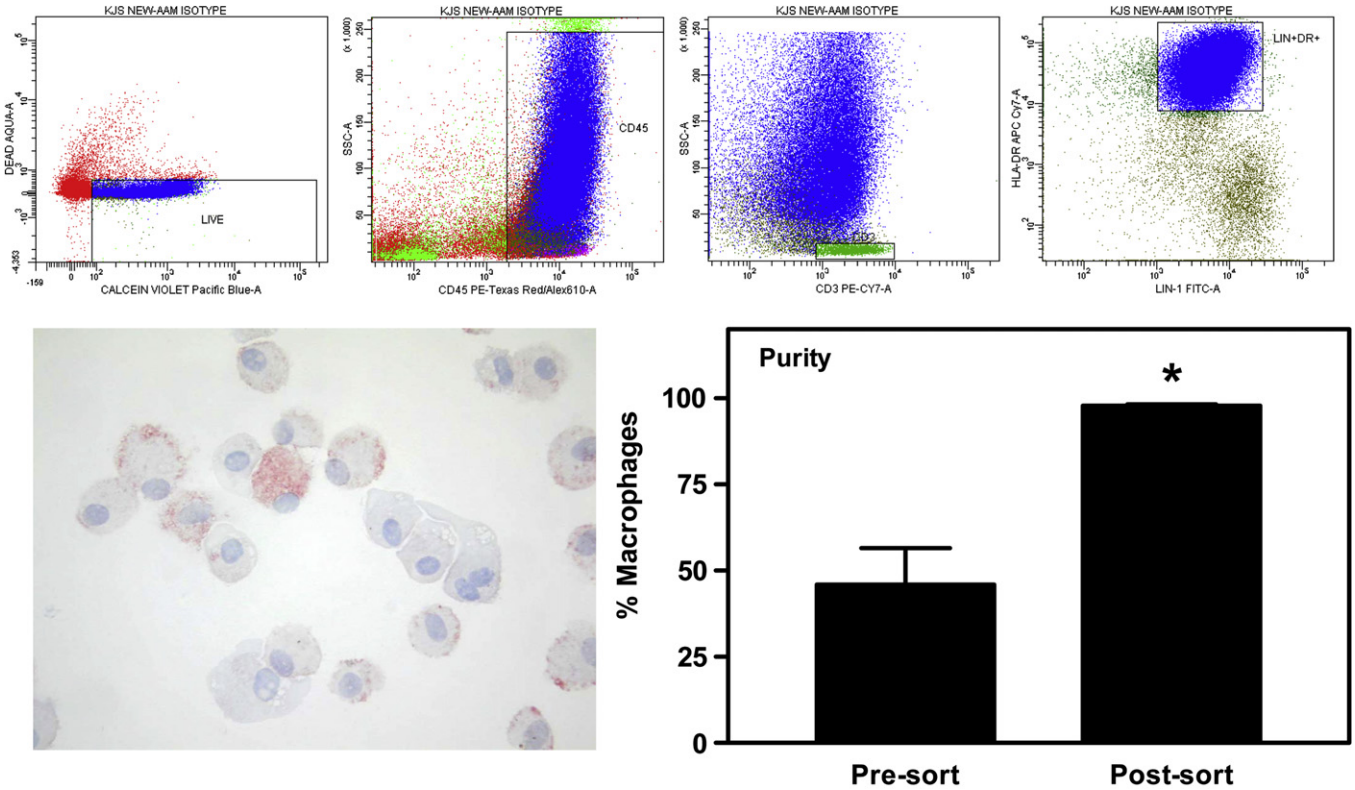

FIG 3. Sorting of lung macrophages for RT-PCR analysis. Sputum and BAL cells were stained according to the method outlined. Live$^+$CD45$^+$CD3$^-$HLA-DR$^+$Lin 1$^+$ events were sorted into PBS with a FACSAria and analyzed by using cytospin preparations (representative micrograph shown at ×40 original magnification). Data are expressed as a percentage of total cells, presented as means ± SEs (n = 6), and analyzed by using the 1-tailed Wilcoxon signed-rank test. *$P < .05$.

were conducted with M2 MDMs, in which fluticasone propionate had no effect on the CCL17 release in response to 10 ng/mL IL-4 (Fig 5, *A*). We then studied whether inhibition of PI3K, the enzyme suggested to be required for differentiation of monocytes into M2 macrophages,[23] could reduce CCL17 release. The pan-PI3K inhibitor LY294002 caused a concentration-dependent inhibition (median effective concentration = 4.2 μM) that was significant at 10 μM (Fig 5, *A*), with no significant lactate dehydrogenase (LDH) release (data not shown). Similar results were obtained with 10 ng/mL IL-13 (data not shown). In contrast, the specific PI3K-δ inhibitor IC81174 had little effect on the amount of CCL17 released by IL-4–stimulated MDMs, except at the highest concentration used (Fig 5, *A*), which caused an increase in LDH release (data not shown). Similarly, the spontaneous release of CCL17 by BAL fluid cells from asthmatic patients (n = 13) was significantly inhibited by 10 μM LY294002 from a mean of 14.82 to 7.49 pg/mL (Fig 5, *B*).

DISCUSSION

In contrast to animal models of asthma, this study has demonstrated only a partial M2 macrophage phenotype in

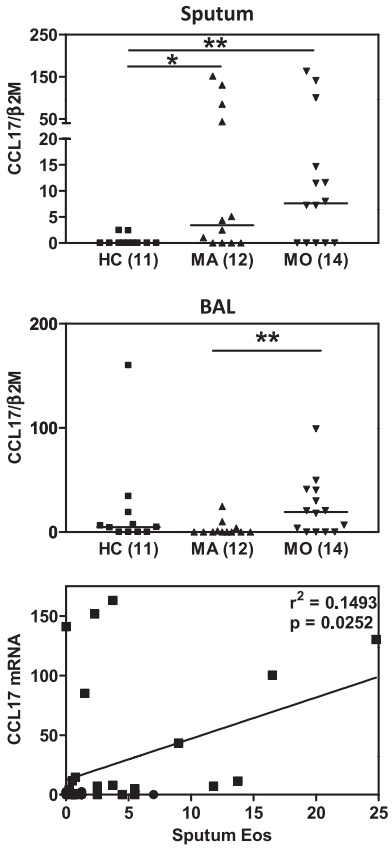

FIG 4. CCL17 mRNA levels (normalized to β_2-microglobulin) and association with sputum eosinophil counts. *Bars* indicate median values, and *numbers in parentheses* indicate *n*. Data were analyzed by using Kruskal-Wallis ANOVA followed by the Dunn multiple comparison test. $*P < .05$ and $**P < .01$. The association between CCL17 mRNA expression and the percentage of sputum eosinophils was analyzed by using the Spearman rank correlation test. *Circles*, Healthy control subjects; *squares*, asthmatic patients.

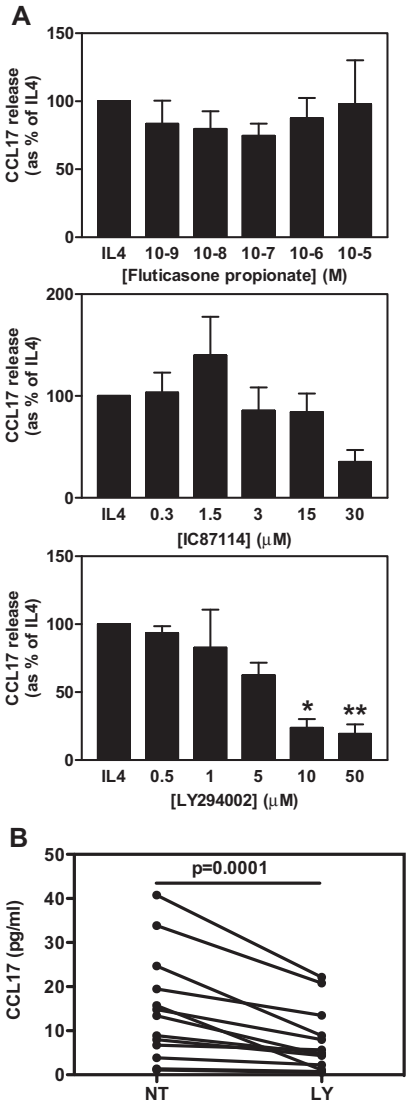

FIG 5. A, Effects of fluticasone propionate and PI3K inhibitors on CCL17 release by IL-4–stimulated MDMs. Data are expressed as a percentage of maximum IL-4 stimulation (mean ± SE, n = 5). Data were analyzed by using Friedman ANOVA and the Dunn multiple comparison test. **B,** Effect of LY294002 on spontaneous release of CCL17 from BAL cells compared to cells not treated *(NT)* (n = 13). Data were analyzed by using the 1-tailed Wilcoxon signed-rank test. $*P < .05$ and $**P < .01$.

airways of asthmatic patients (see Table III), consisting of increased CCL17 mRNA expression and reduced CD163 cell-surface expression when compared with that seen in healthy subjects. Although blood monocytes did not express detectable amounts of CCL17 mRNA (data not shown), increased expression was evident in lung macrophages, regardless of asthma severity and treatment. The expression of CCL17 mRNA in sputum correlated weakly with sputum eosinophilia, a hallmark of asthma and a marker of T_H2-type inflammation,[24] providing a mechanism for increased recruitment of $CCR4^+$ cells (eosinophils and T_H2 lymphocytes) for which CCL17 is a ligand. The finding of persistently increased CCL17 mRNA levels in macrophages from both sputum and BAL of patients with moderate asthma taking inhaled corticosteroids and the inability of corticosteroids to inhibit *in vitro* CCL17 release by M2 MDMs suggest that CCL17 production is relatively corticosteroid insensitive. Finally, our study suggests that the production of CCL17 is regulated by PI3K.

Studies of macrophages in asthma have reported on functional readouts, such as suppression of T-cell proliferation and phagocytic function,[25,26] cell-surface marker expression,[27-29] and gene expression.[30-32] To our knowledge, this is the most comprehensive investigation of M2 lung macrophage biomarkers in asthmatic patients using combined cell-surface and gene expression analysis and measuring cytokine release. Two previous studies, applying gene expression analysis[32] and immunocytochemistry,[29] respectively, showed no evidence for M2 macrophages in asthmatic patients. Another study of M1/M2 macrophages in patients with chronic obstructive pulmonary disease (COPD) using a combination of transcriptomic analysis, RT-PCR, and flow cytometry[33] showed that smoking was associated with increased M2 phenotype gene expression, with further upregulation in smokers with COPD and a reciprocal decrease in M1 phenotype gene expression.[33] However, the profile of differentially expressed M2 markers (eg, adenosine A3 receptor, matrix metalloproteinase [MMP] 2, and MMP7) was different from that seen in our study, whereas CCL17 and CD163 were not differentially expressed in either healthy smokers or smokers with COPD.

Interpretation of the literature is complicated by differences in the definition of M2 phenotypes based on the use of different

TABLE III. Differences in expression of IL-4/IL-13–induced M2 markers in different settings

Marker	Murine MDM2*	Human MDM2*	M2 MDM model in current study†	Asthma macrophages†
Gene expression				
Arg1	↑	No change	No change	No expression
Clec10a§	↑	↑	↑	No change
Fizz1	↑	No human homolog	NT	NT
Ym1	↑	No human homolog	NT	NT
Cell-surface expression				
CD23	↑	↑	NT	NT
CD163	↑[38]	↓[22]	↓	↓
CD206	↑	↑	↑	No change
MHCII/HLA-DR	↑	↑	↑	No change
Chemokine expression				
CCL13‖	↑	↑	NT	NT
CCL14	No murine homolog	↑	NT	NT
CCL17	↑	↑	↑	↑
CCL18	No murine homolog	↑	NT	NT
CCL22	↑	↑	↑‡	No change
CCL24	↑	↑	NT	NT

NT, Not tested.
*Information from Martinez et al,[5] except where referenced differently.
†Data from the current study.
‡Data from the pilot of this article obtained by using a slightly different culture model (see Fig E2 in this article's Online Repository).
§Murine equivalent is MGL1.
‖Murine homolog of CCL13 is CCL2.

M2-polarizing protocols, applying either IL-4/IL-13,[5] IL-10,[7] or macrophage colony-stimulating factor,[34] all of which produce slightly different macrophage phenotypes. Atopic asthma is considered to involve T_H2 cytokines,[2] and therefore in keeping with the M2 phenotype described by Martinez et al,[5] we have used IL-4– and IL-13–stimulated MDMs as our M2 benchmark.

In the current study CCL17 and CD163 expression varied considerably between the subjects studied, which is consistent with the notion that asthma involves heterogeneous inflammatory processes despite similar clinical phenotypes. Differences in CCL17 expression were more pronounced in sputum than in BAL fluid, which are sampled from the central airways and the distal airways and alveoli, respectively,[35] suggesting that the changes reflect the proximal airways processes. Similarly, only the sputum samples showed reduced levels of CD163.

The role of CCL17 in allergic inflammation has been the subject of a number of studies showing increased release after allergen exposure.[12,13,36] Applying flow cytometry to induced sputum and bronchial biopsy explants, we have previously shown an important role for CCR4 and its ligand CCL17 in T_H2 T-cell recruitment.[12] Stimulation of bronchial tissue explants by allergen induced CCL17 release in steroid-naive asthmatic patients, but of more relevance to the current study, spontaneous release was increased in explants from patients with moderate asthma taking inhaled corticosteroids. Furthermore, the numbers of sputum and blood $CCR4^+$ T cells were significantly increased in asthmatic patients taking high-dose inhaled and oral corticosteroids. Taken together with the current findings that CCL17 mRNA expression is persistently high in corticosteroid-treated asthmatic patients and that *in vitro* IL-4–induced CCL17 release by M2 MDMs cannot be suppressed by corticosteroids, this study adds to accumulating evidence that CCL17 is an important chemokine in asthmatic patients, the production of which is relatively corticosteroid insensitive.

Increased expression of CD163, a haptoglobin-hemoglobin scavenger receptor,[37] has been associated with the M2 phenotype *in vitro*.[5,7,10,38] Although most studies showed a role for IL-4 and IL-13 in driving this phenotype, Tiemessen et al[7] showed it to depend on IL-10 and not IL-4/IL-13. In contrast to the published *in vitro* studies, we have found that CD163 expression is decreased in asthmatic patients. The finding that MDMs exposed to IL-4 and IL-13 reduced CD163 expression suggests that T_H2 cytokines might be responsible in asthmatic patients. In agreement with our study, Van den Heuvel et al[22] demonstrated that IL-4 treatment decreases CD163 expression, although when added to cells pretreated with glucocorticoids, it increases the expression. In contrast, in our study the reduced expression of CD163 on sputum macrophages from asthmatic patients was evident despite treatment with inhaled steroids, providing additional evidence of relative corticosteroid insensitivity. Thus our observations are consistent with the notion that airway macrophages are relatively corticosteroid insensitive.[39]

Having found the macrophages to be insensitive to corticosteroids, we sought an alternative therapy targeting PI3K, the enzyme postulated to be required for the differentiation of M2 macrophages.[23] For this purpose, LY294002 was selected on the basis of its relative selectivity for PI3K. At a concentration of 10 μM, this compound was effective at inhibiting CCL17 release from both cultured BAL fluid cells and IL-4–stimulated MDMs. Although this suggests the involvement of PI3K, it is recognized that at concentrations of greater than 10 μM, LY294002 inhibits other kinases.[40] Previous studies have suggested that inhibiting the PI3K-δ isoform is effective at reducing airway inflammation and hyperresponsiveness in murine models of asthma.[41] Furthermore, inhibition of PI3K-δ with theophylline reverses the steroid insensitivity of macrophages from patients with COPD.[42] However, the specific PI3K-δ inhibitor IC87114 had little effect on IL-4–induced CCL17 release from MDMs, suggesting that, in contrast to the murine model,[23] an isoform other than PI3K-δ is involved in IL-4–induced CCL17 production in human macrophages. In summary, this study suggests involvement of the

PI3K pathway, but definitive proof of this being involved will require more specific inhibition using such tools as inhibitory RNAs.

This study raises a number of questions and opportunities for further research. Previous studies[8] have shown that CLEC10A mRNA expression is increased on M2 MDMs, and there have been reports of increases on macrophages in patients with a number of diseases.[43-45] However, to our knowledge, there have been no studies of both mRNA and protein expression. In our study there was a trend toward increased CLEC10A mRNA but not CD301 (the protein marker of CLEC10A) expression in macrophages of asthmatic patients. Future studies will need to look for internalized or intracellular CD301 expression. The difference in CCL17 mRNA expression was greater in sputum than in BAL, and the reasons for that are unclear. This discrepancy could, at least in part, account for the lack of difference in CCL17 protein release by BAL fluid cells *ex vivo*. Other cell types, although they constituted, on average, 30% of total BAL fluid cells, might have bound some of the cytokines/chemokines released in culture (eg, CCL17 by $CCR4^+$ cells). Furthermore, contact with plastic might have altered the BAL macrophage phenotype.[46] Similarly, the *in vivo* conditions required for the phenotype to be detected might not be sustained *ex vivo*. However, it is unlikely that either of these would have offset any intrinsic differences between macrophages from patients and healthy subjects. When studying macrophage function, stimuli are often used, but we chose not to expose the BAL fluid cells to any exogenous stimuli, such as LPS, because this could skew responses toward an M1 profile.[5] Finally, the biomarker panel in this study was limited to biomarkers published at the time the study was conceived; further studies should focus on additional M2 biomarkers, such as MMP2 and MMP7.[33]

The driver of the macrophage phenotype in asthmatic patients remains unclear. It is possible that IL-4 and IL-13 do provoke this phenotype but that our MDM model does not encapsulate the full complexity of macrophage interactions within the lung environment. IL-4/IL-13 and activation of signal transducer and activator of transcription 6 might not be involved in the development of the CCL17-producing macrophages. The promoter for *CCL17* also contains nuclear factor κB (NF-κB) motifs,[47,48] and the archetypal NF-κB activator, TNF, induces expression of CCL17 mRNA in human bronchial epithelial cells.[49] Infection of murine lung epithelium with respiratory syncytial virus activates the NF-κB pathway to produce CCL17[50] and switches macrophages to a M2 phenotype.[51] Moreover, the house dust mite allergen *Dermatophagoides pteronyssinus* (Der p 1), also induces CCL17 mRNA and protein expression in cultured primary bronchial epithelial cells through an epithelial growth factor receptor/NF-κB–dependent pathway.[16] Because both respiratory syncytial virus and Der p 1 are implicated in asthma,[52,53] these agents may activate macrophages through NF-κB–dependent pathways to produce CCL17 in a steroid-independent manner.

This study has involved a mix of eosinophilic and noneosinophilic asthma. The increasing recognition that phenotypes based on eosinophilic and neutrophilic inflammation are different clinically and in respect of response to corticosteroids will require further study of the identified biomarkers in these phenotypes.

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Key messages

- **Lung macrophages in asthmatic patients do not express biomarkers associated with the alternative M2 phenotype reported in studies of MDMs and animal models.**
- **Lung macrophages of asthmatic patients express more CCL17 and less CD163, and the former is associated, although weakly, with sputum eosinophilia and cannot be suppressed by corticosteroids but can be attenuated by PI3K inhibition.**
- **Macrophage expression of CCL17 may contribute to the airway inflammation observed in asthmatic patients through recruitment of cells expressing CCR4, the cell-surface receptor for CCL17.**

REFERENCES

1. Bousquet J, Chanez P, Lacoste JY, Barneon G, Ghavanian N, Enander I, et al. Eosinophilic inflammation in asthma. N Engl J Med 1990;323:1033-9.
2. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992;326:298-304.
3. Robinson D, Hamid Q, Bentley A, Ying S, Kay AB, Durham SR. Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. J Allergy Clin Immunol 1993;92:313-24.
4. Peters-Golden M. The alveolar macrophage: the forgotten cell in asthma. Am J Respir Cell Mol Biol 2004;31:3-7.
5. Martinez FO, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol 2009;27:451-83.
6. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. J Exp Med 1992;176:287-92.
7. Tiemessen MM, Jagger AL, Evans HG, van Herwijnen MJ, John S, Taams LS. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. Proc Natl Acad Sci U S A 2007;104:19446-51.
8. Raes G, Brys L, Dahal BK, Brandt J, Grooten J, Brombacher F, et al. Macrophage galactose-type C-type lectins as novel markers for alternatively activated macrophages elicited by parasitic infections and allergic airway inflammation. J Leukoc Biol 2005;77:321-7.
9. Kurowska-Stolarska M, Stolarski B, Kewin P, Murphy G, Corrigan CJ, Ying S, et al. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. J Immunol 2009;183:6469-77.
10. Prasse A, Germann M, Pechkovsky DV, Markert A, Verres T, Stahl M, et al. IL-10-producing monocytes differentiate to alternatively activated macrophages and are increased in atopic patients. J Allergy Clin Immunol 2007;119:464-71.
11. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. J Leukoc Biol 2011;89:557-63.
12. Vijayanand P, Durkin K, Hartmann G, Morjaria J, Seumois G, Staples KJ, et al. Chemokine receptor 4 plays a key role in T cell recruitment into the airways of asthmatic patients. J Immunol 2010;184:4568-74.
13. Panina-Bordignon P, Papi A, Mariani M, Di Lucia P, Casoni G, Bellettato C, et al. The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. J Clin Invest 2001;107:1357-64.
14. Sekiya T, Miyamasu M, Imanishi M, Yamada H, Nakajima T, Yamaguchi M, et al. Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells. J Immunol 2000;165:2205-13.
15. Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, et al. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. J Immunol 2005;174:8183-90.
16. Heijink IH, Marcel Kies P, van Oosterhout AJ, Postma DS, Kauffman HF, Vellenga E. Der p, IL-4, and TGF-beta cooperatively induce EGFR-dependent TARC expression in airway epithelium. Am J Respir Cell Mol Biol 2007;36:351-9.
17. Perros F, Hoogsteden HC, Coyle AJ, Lambrecht BN, Hammad H. Blockade of CCR4 in a humanized model of asthma reveals a critical role for DC-derived

CCL17 and CCL22 in attracting Th2 cells and inducing airway inflammation. Allergy 2009;64:995-1002.
18. Tudhope SJ, Finney-Hayward TK, Nicholson AG, Mayer RJ, Barnette MS, Barnes PJ, et al. Different mitogen-activated protein kinase-dependent cytokine responses in cells of the monocyte lineage. J Pharmacol Exp Ther 2008;324:306-12.
19. Taylor AE, Finney-Hayward TK, Quint JK, Thomas CM, Tudhope SJ, Wedzicha JA, et al. Defective macrophage phagocytosis of bacteria in COPD. Eur Respir J 2010;35:1039-47.
20. Vijayanand P, Seumois G, Pickard C, Powell RM, Angco G, Sammut D, et al. Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. N Engl J Med 2007;356:1410-22.
21. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001;25:402-8.
22. Van den Heuvel MM, Tensen CP, van As JH, Van den Berg TK, Fluitsma DM, Dijkstra CD, et al. Regulation of CD 163 on human macrophages: cross-linking of CD163 induces signaling and activation. J Leukoc Biol 1999;66:858-66.
23. Rauh MJ, Ho V, Pereira C, Sham A, Sly LM, Lam V, et al. SHIP represses the generation of alternatively activated macrophages. Immunity 2005;23:361-74.
24. Pizzichini E, Pizzichini MM, Efthimiadis A, Evans S, Morris MM, Squillace D, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. Am J Respir Crit Care Med 1996;154:308-17.
25. Spiteri MA, Knight RA, Jeremy JY, Barnes PJ, Chung KF. Alveolar macrophage-induced suppression of peripheral blood mononuclear cell responsiveness is reversed by in vitro allergen exposure in bronchial asthma. Eur Respir J 1994;7:1431-8.
26. Fitzpatrick AM, Holguin F, Teague WG, Brown LA. Alveolar macrophage phagocytosis is impaired in children with poorly controlled asthma. J Allergy Clin Immunol 2008;121:1372-8, e1-3.
27. Viksman MY, Liu MC, Bickel CA, Schleimer RP, Bochner BS. Phenotypic analysis of alveolar macrophages and monocytes in allergic airway inflammation. I. Evidence for activation of alveolar macrophages, but not peripheral blood monocytes, in subjects with allergic rhinitis and asthma. Am J Respir Crit Care Med 1997;155:858-63.
28. Lensmar C, Katchar K, Eklund A, Grunewald J, Wahlstrom J. Phenotypic analysis of alveolar macrophages and lymphocytes following allergen inhalation by atopic subjects with mild asthma. Respir Med 2006;100:918-25.
29. St-Laurent J, Turmel V, Boulet LP, Bissonnette E. Alveolar macrophage subpopulations in bronchoalveolar lavage and induced sputum of asthmatic and control subjects. J Asthma 2009;46:1-8.
30. Baines KJ, Simpson JL, Wood LG, Scott RJ, Gibson PG. Transcriptional phenotypes of asthma defined by gene expression profiling of induced sputum samples. J Allergy Clin Immunol 2011;127:153-60, e1-9.
31. Madore AM, Perron S, Turmel V, Laviolette M, Bissonnette EY, Laprise C. Alveolar macrophages in allergic asthma: an expression signature characterized by heat shock protein pathways. Hum Immunol 2010;71:144-50.
32. Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, et al. T-helper type 2-driven inflammation defines major subphenotypes of asthma. Am J Respir Crit Care Med 2009;180:388-95.
33. Shaykhiev R, Krause A, Salit J, Strulovici-Barel Y, Harvey BG, O'Connor TP, et al. Smoking-dependent reprogramming of alveolar macrophage polarization: implication for pathogenesis of chronic obstructive pulmonary disease. J Immunol 2009;183:2867-83.
34. Leidi M, Gotti E, Bologna L, Miranda E, Rimoldi M, Sica A, et al. M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells in vitro. J Immunol 2009;182:4415-22.
35. Alexis NE, Hu SC, Zeman K, Alter T, Bennett WD. Induced sputum derives from the central airways: confirmation using a radiolabeled aerosol bolus delivery technique. Am J Respir Crit Care Med 2001;164:1964-70.
36. Bochner BS, Hudson SA, Xiao HQ, Liu MC. Release of both CCR4-active and CXCR3-active chemokines during human allergic pulmonary late-phase reactions. J Allergy Clin Immunol 2003;112:930-4.
37. Van Gorp H, Delputte PL, Nauwynck HJ. Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. Mol Immunol 2010;47:1650-60.
38. Gordon S. Alternative activation of macrophages. Nat Rev Immunol 2003;3:23-35.
39. Bhavsar P, Hew M, Khorasani N, Torrego A, Barnes PJ, Adcock I, et al. Relative corticosteroid insensitivity of alveolar macrophages in severe asthma compared with non-severe asthma. Thorax 2008;63:784-90.
40. Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, et al. The selectivity of protein kinase inhibitors: a further update. Biochem J 2007;408:297-315.
41. Lee KS, Lee HK, Hayflick JS, Lee YC, Puri KD. Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. FASEB J 2006;20:455-65.
42. To Y, Ito K, Kizawa Y, Failla M, Ito M, Kusama T, et al. Targeting phosphoinositide-3-kinase-delta with theophylline reverses corticosteroid insensitivity in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 2010;182:897-904.
43. Prokop S, Heppner FL, Goebel HH, Stenzel W. M2 polarized macrophages and giant cells contribute to myofibrosis in neuromuscular sarcoidosis. Am J Pathol 2011;178:1279-86.
44. Desai B, Mattson J, Paintal H, Nathan M, Shen F, Beaumont M, et al. Differential expression of monocyte/macrophage-selective markers in human idiopathic pulmonary fibrosis. Exp Lung Res 2011;37:227-38.
45. Semnani RT, Mahapatra L, Moore V, Sanprasert V, Nutman TB. Functional and phenotypic characteristics of alternative activation induced in human monocytes by interleukin-4 or the parasitic nematode *Brugia malayi*. Infect Immun 2011;79:3957-65.
46. Sikkeland LI, Kongerud J, Stangeland AM, Haug T, Alexis NE. Macrophage enrichment from induced sputum. Thorax 2007;62:558-9.
47. Wirnsberger G, Hebenstreit D, Posselt G, Horejs-Hoeck J, Duschl A. IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. Eur J Immunol 2006;36:1882-91.
48. Komine M, Kakinuma T, Kagami S, Hanakawa Y, Hashimoto K, Tamaki K. Mechanism of thymus- and activation-regulated chemokine (TARC)/CCL17 production and its modulation by roxithromycin. J Invest Dermatol 2005;125:491-8.
49. Berin MC, Eckmann L, Broide DH, Kagnoff MF. Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. Am J Respir Cell Mol Biol 2001;24:382-9.
50. Monick MM, Powers LS, Hassan I, Groskreutz D, Yarovinsky TO, Barrett CW, et al. Respiratory syncytial virus synergizes with Th2 cytokines to induce optimal levels of TARC/CCL17. J Immunol 2007;179:1648-58.
51. Shirey KA, Pletneva LM, Puche AC, Keegan AD, Prince GA, Blanco JC, et al. Control of RSV-induced lung injury by alternatively activated macrophages is IL-4R alpha-, TLR4-, and IFN-beta-dependent. Mucosal Immunol 2010;3:291-300.
52. Sigurs N, Aljassim F, Kjellman B, Robinson PD, Sigurbergsson F, Bjarnason R, et al. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. Thorax 2010;65:1045-52.
53. Pilette C, Francis JN, Till SJ, Durham SR. CCR4 ligands are up-regulated in the airways of atopic asthmatics after segmental allergen challenge. Eur Respir J 2004;23:876-84.

METHODS

Monocyte isolation and differentiation into M2 macrophages

PBMCs from healthy control subjects and asthmatic patients were isolated from heparinized blood by means of centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Monocytes were then isolated from the PBMCs by using $CD14^+$ microbeads (Miltenyi Biotec, Bisley, United Kingdom), according to the manufacturer's instructions. Isolated monocytes were resuspended in RPMI supplemented with 10% heat-inactivated FBS, 2 mg/ml L-glutamine, 0.05 U/mL penicillin, 50 μg/mL streptomycin, and 0.5 mg/mL amphotericin B (all from Invitrogen).

In preliminary experiments leading to this work, monocytes were cultured in 20 ng/mL IFN-γ or IL-4 (both from R&D Systems, Abingdon, United Kingdom). After 72 hours, cells were harvested for flow cytometry and gene expression analysis (Fig E2).

For the main body of work, monocytes were differentiated into macrophages over 12 days in the presence of 2 ng/mL GM-CSF (R&D Systems), according to the method of Tudhope et al.[E1] MDMs were then washed extensively with basal RPMI before addition of RPMI supplemented as above without GM-CSF followed by addition of IL-4 or IL-13 (10 ng/mL, both from R&D Systems) for 24 hours to polarize them toward a M2 phenotype. For flow cytometric analysis, MDMs generated were removed from culture plates by using a nonenzymatic cell dissociation solution (Sigma, Poole, United Kingdom) before resuspending in FACS buffer (PBS, 0.5% wt/vol BSA, and 2 mM EDTA) containing 2 mg/mL human IgG (Sigma). For RNA isolation, Stratagene lysis buffer was added directly to the plates after removal of supernatants. Lysis buffer was then removed to Eppendorf tubes before storage at −80°C. Although MDMs were generated from a mixture of asthmatic patients (n = 6) and healthy control subjects (n = 5), no significant difference in IL-4–induced expression of CCL17 and CLEC10A steady-state mRNA was observed between these 2 groups (data not shown).

Flow cytometric analysis of MDMs

For phenotypic characterization of cytokine-stimulated MDMs, cells resuspended in FACS buffer were stained with mixtures of APC-Cy7–labeled anti–HLA-DR (BD Biosciences), PerCP-Cy5.5–labeled anti-CD14 (BD Biosciences), and AF647-labeled anti-CD301 (Cambridge Bioscience, Cambridge, United Kingdom); mixtures of APC-labeled anti-CD206 (BD Biosciences), PerCP-Cy5.5-labeled anti-CD163 (Cambridge Bioscience), and fluorescein isothiocyanate–labeled anti-CD36 (BD Biosciences); or appropriate isotype controls for 30 minutes on ice. Analysis was performed on a 9-color FACSAria cell sorter (BD Biosciences) with FACSDiva Software (version 5.0.3).

Collection of lung cell samples

Sputum induction and solubilization were performed as previously described.[E2] Fiberoptic bronchoscopy was carried out in accordance with the recommendations of the American Thoracic Society.[E3] One hundred twenty milliliters of normal saline was instilled into the right upper lobe and recovered by means of aspiration. Cell aliquots were immediately resuspended in FACS buffer containing 2 mg/mL human IgG or cultured in AIM V media supplemented with 0.05 U/mL penicillin, 50 μg/mL streptomycin, 0.5 mg/mL amphotericin B, 1 mmol/L sodium pyruvate and 0.004% (vol/vol) 2-mercaptoethanol (all from Invitrogen) at a concentration of 1×10^6 cells/mL for 24 hours.

Flow cytometry of lung macrophage biomarkers

Phenotypic characterization of lung macrophages was performed by using flow cytometry with a 9-color FACSAria cell sorter (BD Biosciences) and FACSDiva Software (version 5.0.3). Sputum and BAL fluid cells were incubated in reagents from the Live/Dead Violet Viability/Vitality kit and the macrophage detection cocktail, consisting of anti-human CD45 PE-AF610 (Invitrogen), anti-human CD3 PE-Cy7 (BD Biosciences), and anti-human HLA-DR APC-Cy7. By using this protocol, $CD45^+CD3^-HLA\text{-}DR^+$ cells were shown to be composed of greater than 90% pure macrophages, as shown by using cytologic analysis of sorted cells (Fig E1).

Separate aliquots were additionally incubated in a cocktail of antibodies against M2 surface markers (anti-CD206 APC and anti-CD14 PerCP-Cy5.5 or anti-CD301 AF647 and anti-CD163 PerCP-Cy5.5) or isotype control antibodies. CD206 and CD163 are expressed as percentages of cells because in most cases there was a clear positive and negative population, whereas CD14 and CD301 are represented as the specific mean fluorescence intensity because the entire selected population stained positive compared with the isotype control.

For intracellular staining, separate aliquots of cells that had been incubated in the macrophage identification cocktail were first washed with FACS buffer and then incubated in Cytofix/Cytoperm (BD). These cells were further washed in 1× Perm/Wash and incubated with anti-human CD68 PE (BD), anti-human TNF APC (eBioscience, San Diego, Calif), anti–IL-10 AF647 (BioLegend, San Diego, Calif), or isotype control. Intracellular cytokine staining is represented as the specific mean fluorescence intensity because the entire selected population stained positive compared with the isotype control.

The 90% purity of macrophages obtained by using the above method of selection was considered insufficient for gene expression analysis because any small contamination with other cell types could be amplified during PCR. Therefore we added the Lin 1 antibody cocktail (BD Biosciences) to the macrophage detection protocol, which made it possible to exclude dendritic cells from the analysis. Cells sorted in this way ($CD45^+CD3^-Lin^+HLA\text{-}DR^+$, Fig 3) from both sputum and BAL fluid were, on average, 98% pure macrophages, as ascertained based on histochemical and morphologic analysis of cytospin preparations. When theses slides were stained for CD68 by using immunocytochemistry, a slightly lower percentage of the sorted cells (91%) were $CD68^+$, with some cells that were morphologically clearly of the macrophage phenotype not staining positively. Up to 100,000 events were sorted into RNA lysis buffer and stored at −80°C before RNA extraction and RT-PCR.

RNA isolation and RT-PCR

RNA was isolated with a Stratagene Microprep Kit. Reverse transcription was carried out with a PrimerDesign nanoScript Reverse Transcriptase kit. PCR amplifications were performed on a BioRad iCycler by using Precision 2X qPCR Mastermix with or without SYBR Green and the following primers (all from PrimerDesign): Indoleamine 2,3-dioxygenase (INDO), forward 5′- TGT GGC AGC AAC TAT TAT AAG ATG -3′ and reverse 5′- CAC TTC TTC ATC AAT ATG GTA CTC TT -3′; CCR7, forward 5′- AAG CCT GGT TCC TCC CTA TC -3′ and reverse 5′- ATG GTC TTG AGC CTC TTG AAA TA -3′; CXCL10, forward 5′- CAG AGG AAC CTC CAG TCT CAG -3′ and reverse 5′- GGT ACT CCT TGA ATG CCA CTT A -3′; ARG1, forward 5′- ATCCC-TAATGACAGTCCCTTTC -3′ and reverse 5′- GCTGATTCTTCCGTTCTT CTTG -3′; CCL17, forward 5′- GAGCCATTCCCCTTAGAAAGC -3′ and reverse 5′- GCATTCTTCACTCTCTTGTTGTT -3′; CCL22 forward 5′- AAT TTG CCC AGA TTC ACC TTT C -3′ and reverse 5′- AAT AAG CCA AGA CCA CAC CAT T -3′; and CLEC10A forward 5′- AGCTGAGGTG-GAGGGTTTC -3′ and reverse 5′- GTGTGCCTTCTGCGTAGTG-3′.

Gene expression was normalized to the relative concentration of the housekeeping gene β_2-microglobulin (designed and validated by PrimerDesign), as determined in a separate PCR.

Cell (MDMs and BAL) culture and supernatant analyses

MDMs generated from monocytes obtained from 5 subjects were cultured in RPMI-1640 (+10% FBS) for 24 hours and stimulated with 10 ng/mL IL-4 in the presence or absence of a range of concentrations of fluticasone propionate (Sigma) or PI3K inhibitors (Calbiochem, Feltham, United Kingdom). Cell supernatants were harvested, and CCL17 concentrations were measured by using ELISA, according to the manufacturer's instructions (R&D Systems). LDH release was measured with the CytoTox 96 Non-Radioactivity Cytotoxicity Assay (Promega, Southampton, United Kingdom), according to the manufacturer's instructions.

Supernatants from unstimulated BAL cells cultured in AIM V medium for 24 hours, comprising median 75% macrophages, 6.4% neutrophils, 2.5% eosinophils, 1% lymphocytes, and 15% epithelial cells, were analyzed by

using the Luminex assay (Bio-Rad) to determine the concentrations of multiple cytokines (IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α, IFN-γ, and CCL22), according to the manufacturer's instructions. Briefly, recombinant cytokines for standard curve generation were purchased from R&D Systems. Capture and detection antibodies were from R&D Systems (TNF-α, macrophage-derived chemokine, and thymus and activation-regulated chemokine), BD Pharmingen (Oxford, United Kingdom) (IL-2, IL-4, and IL-13 capture antibodies and IL-5, IL-10, IL-12p70, and IL-13 detection antibodies), Endogen (Loughborough, United Kingdom) (IFN-γ, IL-6, IL-8, IL-10, and IL-12p70 capture antibodies and IFN-γ, IL-4, IL-6, IL-8, and IL-12p70 detection antibodies), and GlaxoSmithKline (Stevenage, United Kingdom; IL-5 capture antibody). Briefly, 50 μL of standards or samples was placed on a 96-well filter plate (Millipore, Watford, United Kingdom) prewetted with 50 μL of assay buffer (PBS, 1% BSA, and 0.025% Triton-X100). Fifty microliters of appropriate antibody-conjugated xMAP Carboxylated Microspheres (Applied Cytometry Systems, Sheffield, United Kingdom) was added to each well (1×10^5 microspheres/mL for each conjugated microsphere set), and the plate was covered and incubated at 4°C overnight. After 2 washes with wash buffer (PBS and 0.05% Tween-20), 50 μL of a cocktail of appropriate detection antibodies (500 ng/mL for each antibody) was added to each well and incubated on a shaker at 700 rpm for 1 hour at room temperature. After 2 further washes, 50 μL of streptavidin-PE (1 μg/mL in assay buffer, BD Biosciences) was incubated on a shaker for 30 minutes at room temperature at 750 rpm. The plate was then washed twice, and 120 μL of sheath fluid was added to each well before reading on a Luminex xMAP100 machine (Bio-Rad). Results were analyzed with STarStation 2.0 software (Applied Cytometry Systems).

RESULTS

Identification of lung macrophages using flow cytometry

Initial identification of macrophages used the specific macrophage marker CD68. However, CD68 is an intracellular marker that can only be detected after fixation and permeabilization of cells. To sort live cells for gene expression studies, surface markers were required to identify macrophages. $CD68^+$ macrophages were therefore identified as being $CD45^+CD3^-HLA\text{-}DR^+$ events (Fig E1, *A*). To corroborate that the combination of $CD45^+CD3^-HLA\text{-}DR^+$ cells was suitable for identifying macrophages, this cocktail of antibodies was combined with a Live/Dead stain (Invitrogen), and $Live^+CD45^+CD3^-HLA\text{-}DR^+$ events were sorted and centrifuged onto cytospin slides. Histochemical and morphologic analysis of the subsequent slides revealed that cells sorted by using this method were 90% macrophages (sputum, ± 5.4% [n = 3], BAL fluid, ± 4.2% [n = 5]) compared with 26% macrophages in presorted sputum (± 7.5% [n = 3]) and 83% macrophages in presorted BAL fluid (± 3.8% [n = 5], Fig E1, *B*).

In previous studies alveolar macrophages have been identified by using flow cytometry on the basis of high autofluorescence in the FL1 and FL2 channels (corresponding to fluorescein isothiocyanate and PE, respectively) when excited by a 488-nm laser,[E4,E5] and this was a particular problem with early single-laser, 2-color flow cytometers. Some investigators have tried to mitigate the effects of autofluorescence by quenching with crystal violet,[E6] whereas others used this autofluorescence to successfully sort pure populations of macrophages.[E7] More recently, analysis has progressed because of the introduction of different dyes that emit peak fluorescence outside of the autofluorescence window (eg, PerCP-Cy5 and APC) and flow cytometers that incorporate more than 1 laser.[E8,E9] We are fortunate to have a 3-laser FACSAria in our division, which allows simultaneous detection of 9 different fluorochromes. Furthermore, this instrument incorporates fiberoptic pathways for collection of emitted light, which, in combination with the band-pass filters in front of the detectors, allows better discrimination of selected wavelengths. In our study we also used the BD standard automatic compensation protocol to reduce the amount of observed autofluorescence to a minimum. Thus when we analyzed BAL fluid cells using flow cytometry, the median percentage of $Live^+$ events that exhibited FL1:FL2 autofluorescence was 3.5%, whereas the median percentage of macrophages in those samples was 79.4% (Fig E1, *C*), demonstrating that autofluorescence of the alveolar macrophages did not interfere with our investigations.

Identification of CCL17 and CLEC10A mRNA expression in BAL fluid macrophages from airways of asthmatic patients

To ascertain a signature associated with an M1 or M2 phenotype, we initially stimulated $CD14^+$ isolated monocytes in the presence of IFN-γ or IL-4 (both at 20 ng/mL), respectively, for 3 days and analyzed the expression of surface receptors and genes (Fig E2) previously identified as markers of these phenotypes (M1: CXCL10, CCR7, and INDO; M2: CLEC10A, CCL17, and CCL22). As previously described, IFN-γ causes increases in surface expression of CD14 but decreases in expression of CD206, whereas IL-4 increases CD206 expression but decreases CD14 expression. At the RNA level, IL-4 caused monocytes to express increased steady-state levels of CLEC10A, CCL17, and CCL22, whereas IFN-γ only significantly increased expression of CXCL10.

To validate this MDM-derived gene signature in lung macrophages, we analyzed the expression of this same panel of genes in macrophages sorted from BAL fluid using a separate cohort of 4 asthmatic volunteers and 4 healthy control subjects. As in the case of MDMs, this analysis also showed undetectable expression of INDO. Although the other genes tested first in MDMs were detectable, CXCL10, CCR7, and CCL22 were not different between macrophages from asthmatic and healthy subjects, and only CLEC10A and CCL17 seemed to be clearly differentially expressed between asthmatic patients and healthy subjects in this pilot cohort of volunteers (Fig E3). Therefore analysis of these 2 genes was taken forward on more subjects.

Ex vivo release of chemokines and cytokines by BAL cells

To ascertain whether increased expression of CCL17 mRNA from lung macrophages was translated into increased release of this chemokine, freshly isolated BAL cells were incubated for 24 hours, and supernatants were harvested to analyze CCL17 release by using the Luminex assay; additional cytokines were also assayed. Only TNF, IL-6, IL-8, IL-10, CCL17, and CCL22 from the Luminex panel were released at detectable levels, with no significant difference between subject groups in the amounts released (Fig E4).

REFERENCES

E1. Tudhope SJ, Finney-Hayward TK, Nicholson AG, Mayer RJ, Barnette MS, Barnes PJ, et al. Different mitogen-activated protein kinase-dependent cytokine responses in cells of the monocyte lineage. J Pharmacol Exp Ther 2008;324:306-12.
E2. Vijayanand P, Seumois G, Pickard C, Powell RM, Angco G, Sammut D, et al. Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. N Engl J Med 2007;356:1410-22.

E3. Djukanovic R, Wilson JW, Lai CK, Holgate ST, Howarth PH. The safety aspects of fiberoptic bronchoscopy, bronchoalveolar lavage, and endobronchial biopsy in asthma. Am Rev Respir Dis 1991;143:772-7.
E4. Skold CM, Hed J, Eklund A. Smoking cessation rapidly reduces cell recovery in bronchoalveolar lavage fluid, while alveolar macrophage fluorescence remains high. Chest 1992;101:989-95.
E5. Viksman MY, Liu MC, Schleimer RP, Bochner BS. Application of a flow cytometric method using autofluorescence and a tandem fluorescent dye to analyze human alveolar macrophage surface markers. J Immunol Methods 1994;172:17-24.
E6. Lofdahl JM, Wahlstrom J, Skold CM. Different inflammatory cell pattern and macrophage phenotype in chronic obstructive pulmonary disease patients, smokers and non-smokers. Clin Exp Immunol 2006;145:428-37.
E7. Maus U, Rosseau S, Seeger W, Lohmeyer J. Separation of human alveolar macrophages by flow cytometry. Am J Physiol Lung Cell Mol Physiol 1997;272: L566-71.
E8. Viksman MY, Liu MC, Bickel CA, Schleimer RP, Bochner BS. Phenotypic analysis of alveolar macrophages and monocytes in allergic airway inflammation. I. Evidence for activation of alveolar macrophages, but not peripheral blood monocytes, in subjects with allergic rhinitis and asthma. Am J Respir Crit Care Med 1997;155:858-63.
E9. van Rijt LS, Kuipers H, Vos N, Hijdra D, Hoogsteden HC, Lambrecht BN. A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. J Immunol Methods 2004;288:111-21.

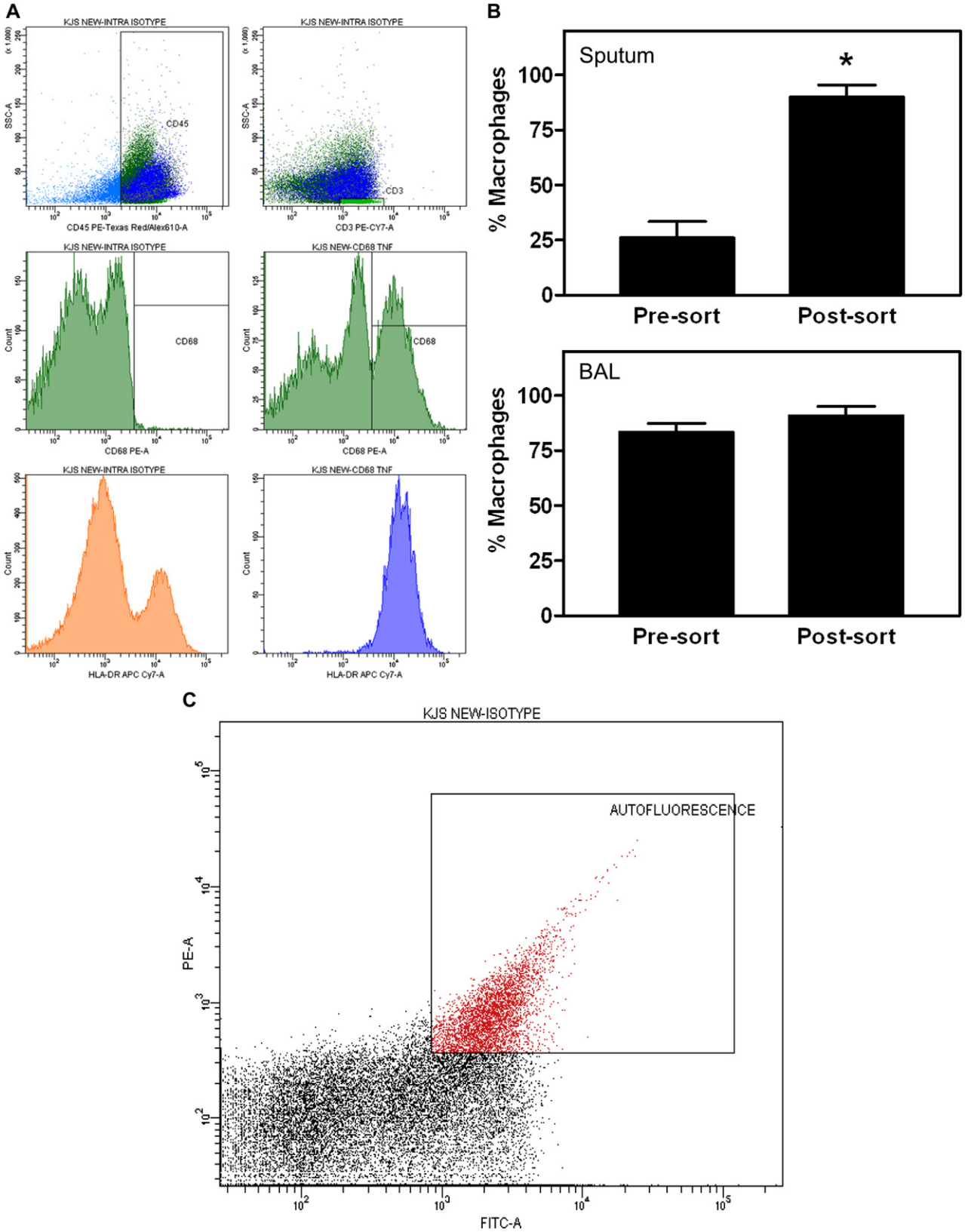

FIG E1. Analysis of lung macrophage M2 marker expression by means of flow cytometry. **A,** Cell pellets from sputum and BAL were incubated with Aqua Dead Dye (Invitrogen) and the antibodies PE-AF610–labeled anti-CD45, PE-Cy7–labeled anti-CD3, and APC-Cy7–labeled anti–HLA-DR or appropriate isotype controls. After fixation and permeabilization, cells were incubated with PE-labeled anti-CD68 or an appropriate isotype control. Backgating of $CD45^{+}CD3^{-}CD68^{+}$ events demonstrates that all these

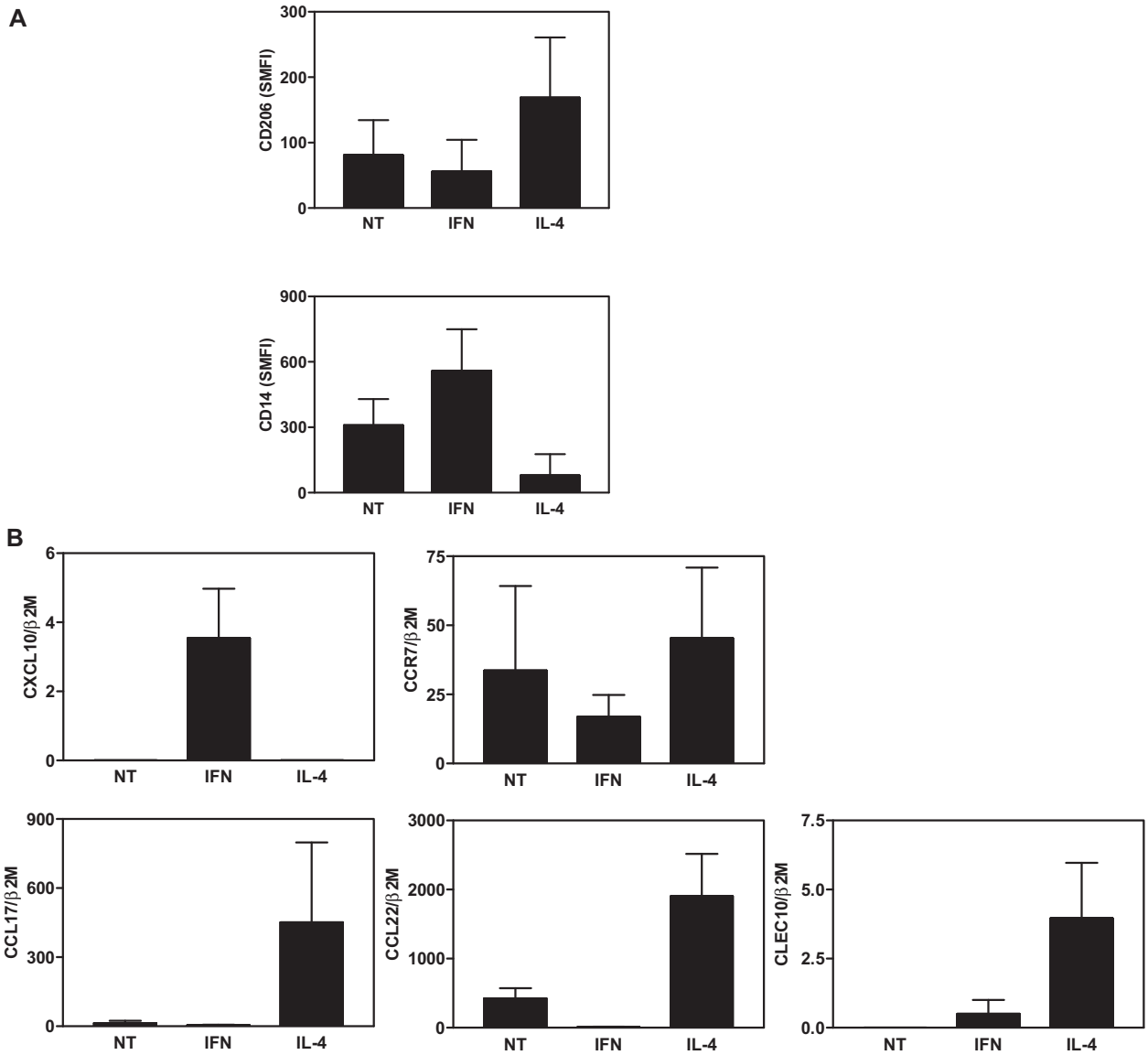

FIG E2. Human monocytes respond to T_H2 cytokines. **A,** Monocytes were stimulated with IL-4 (20 ng/mL) for 24 hours and analyzed for cell-surface expression of CD14 and CD206 by means of flow cytometry. Data are expressed as mean fluorescence intensity corrected for isotype control staining *(SMFI)* and presented as means ± SEs (n = 4). **B,** Gene expression of M1 or M2 biomarkers was analyzed by means of RT-PCR. Data were normalized to β_2-microglobulin *(β2M)* and are expressed as means ± SEs (n = 4). *NT,* Not treated.

cells express high levels of HLA-DR. Plots are representative of 10 independent sputum samples. **B,** Live$^+$CD45$^+$CD3$^-$HLA-DR$^+$ events were sorted into PBS by using a FACSAria, and the selected cells were loaded onto a cytospin and stained with Diff-Quick. Cells were counted, and data are expressed as a percentage of nonsquamous cells and presented as means ± SEs (sputum, n = 3; BAL, n = 5). Data were further analyzed by using the 1-tailed Mann-Whitney *U* test. $*P < .05$ compared with the presort sample. **C,** Representative scatter plot showing low fluorescein isothiocyanate *(FITC)*/PE autofluorescence of Live$^+$ events from BAL samples.

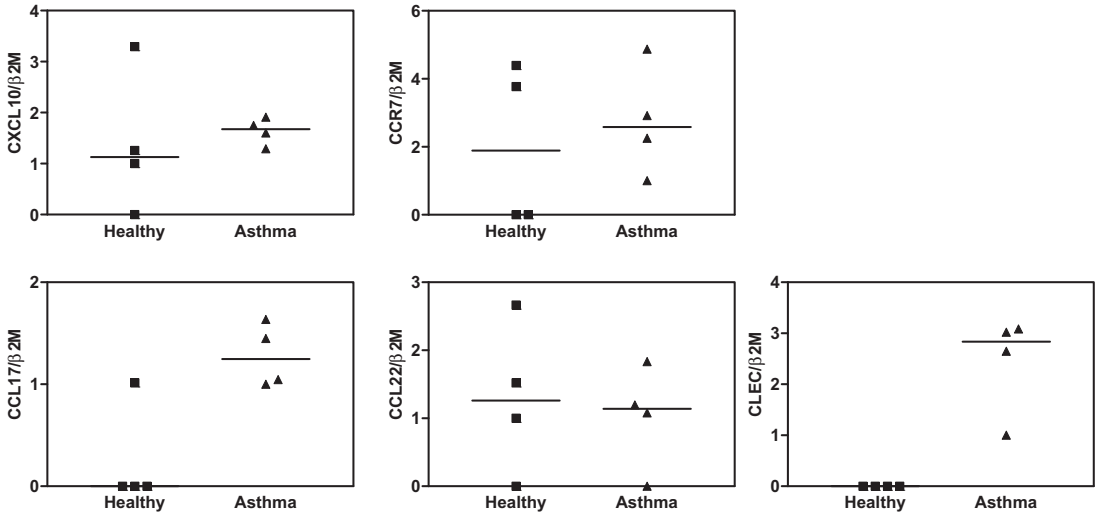

FIG E3. Preliminary analysis of BAL macrophage gene expression. Live$^+$CD45$^+$CD3$^-$HLA-DR$^+$Lin 1$^+$ events from BAL of 4 healthy control subjects and 4 asthmatic patients were sorted into lysis buffer, and RNA was extracted and reverse transcribed. Steady-state levels of gene expression were measured by using real-time PCR and normalized to β_2-microglobulin *(β2M). Bars* indicate median expression relative to β_2M.

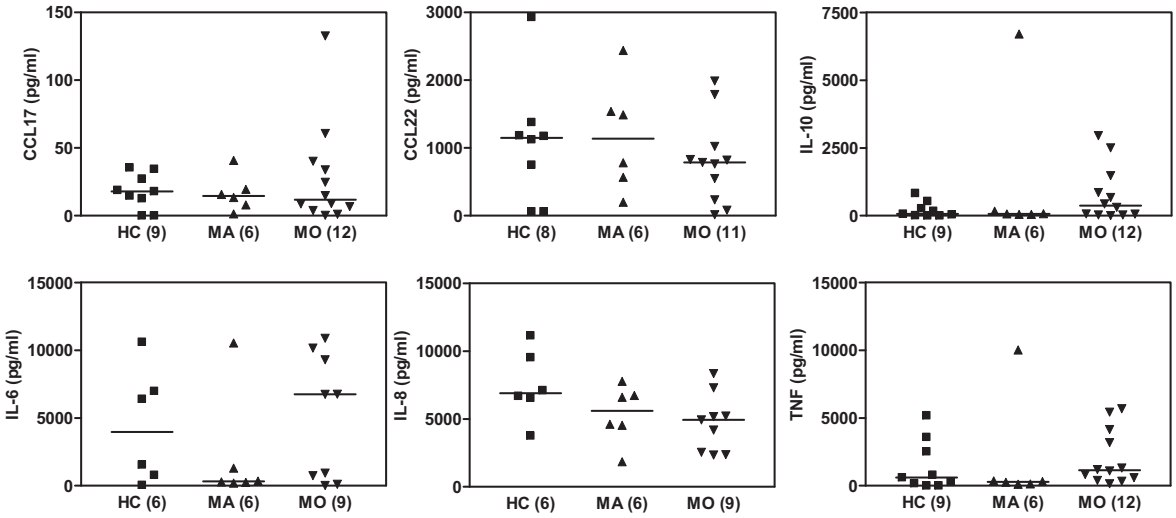

FIG E4. *Ex vivo* release of cytokines by BAL cells from healthy control subjects *(HC)*, patients with mild asthma *(MA)*, or patients with moderate asthma *(MO)*. Spontaneous release of cytokines into supernatants after 24 hours in cell culture was assessed by using the Luminex assay. *Bars* indicate median values, and data were analyzed with a Kruskal-Wallis ANOVA followed by the Dunn multiple comparison test.