Phenotypic characterisation of lung macrophages in asthma: over-expression of CCL17

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Abstract

Background—Studies using monocyte-derived macrophages (MDM) 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—Phenotypically to characterize lung macrophages from asthmatic patients in relation to disease severity and treatment.

Methods—M2 biomarkers were first identified using MDM exposed to Th2 cytokines and then used to phenotype sputum and bronchoalveolar lavage (BAL) macrophages from 12 healthy controls, 12 mild and 14 moderate asthmatics and to assess the effects of corticosteroids and phosphatidylinositol-3-kinase (PI3K) inhibitors.

Results—Sputum macrophages from asthmatics expressed significantly more CCL17 mRNA but less CD163 than macrophages from healthy individuals. However, none of the other M2 biomarkers were differentially expressed in asthma and *ex vivo* BAL cells spontaneously produced similar amounts of M2 cytokine/chemokines (IL-10, CCL17 and CCL22). CCL17 mRNA over-expression correlated weakly but significantly with sputum eosinophilia (p=0.0252) and was also observed in macrophages from moderate asthmatics treated with inhaled steroids, suggesting relative insensitivity to inhibition by corticosteroids. The PI3Kinase inhibitor LY294002 inhibited basal CCL17 release from BAL cells and IL-4-stimulated release from MDM.

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 mild and moderate asthma, providing a further source for this ligand of CCR4+ cells that contribute to airways inflammation. CCL17 expression is corticosteroid resistant but is suppressed by PI3Kinase enzyme inhibitors.

Introduction

Asthma is a complex airways inflammatory disease involving several cell types, with most research focusing on eosinophils and CD4+ T helper (Th)-2 cells[1, 2], the latter being an

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important source of cytokines IL-4, IL-5 and IL-13, key drivers of responses to allergen[3]. The role of macrophages in driving allergic airways disease has been largely overlooked[4] even though they are the prevalent immune cell type in the lungs.

Macrophages have been broadly characterised 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 (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 Th2 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 recognised that there are differences between human and murine M2 expression profiles[11].

Given that macrophages are the most numerous inflammatory cell in the airways where Th2 cytokines are increased, we postulated that lung macrophages from asthmatics are of the M2 phenotype. We also hypothesised 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 Th2 cytokines[12] and that their recruitment into asthmatic airways 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 macrophages derived from monocytes (MDM) cultured in M2-polarising conditions; these biomarkers were then used to phenotype sputum and BAL macrophages from mild, steroid-naive and moderate, steroid-treated asthmatics and nonatopic controls. In addition to studying M1 (CD14, TNF) and M2 (*Ccl17, Clec10a, Arg1*, IL-10, IL-13, CD163, CD206, CLEC10A/CD301) biomarkers at the level of mRNA, intracellular protein and cell surface expression we also assessed the potential of airway macrophages recovered by BAL to produce a series of cytokines/chemokines 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, IL-10) phenotypes[5]. Having found that the frequency of lung macrophages expressing CCL17 is increased in both steroid-naive and steroid-treated asthmatics, we studied the effects of the corticosteroid, fluticasone propionate, and inhibitors of phosphatidylinositol-3-kinase (PI3Kinase) on *ex vivo* CCL17 production by MDM and BAL macrophages to explore their therapeutic potential.

Methods

Subjects

12 mild atopic asthmatics taking short-acting -agonists alone (MA), 14 moderate atopic asthmatics requiring inhaled corticosteroids for disease control (MO), classified according to GINA criteria (www.ginasthma.org), and 12 healthy non-atopic control subjects (HC) were studied (Table 1). All subjects were non-smokers with no respiratory infections for 6 weeks prior to the study. Atopy was assessed 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 using macrophages derived from blood monocytes

In order to establish a panel of biomarkers that reflect M2 macrophages for subsequent phenotyping of lung macrophages from asthmatic and control subjects, initial experiments were conducted on MDMs derived from circulating monocytes. Monocytes were isolated to >95% purity by magnetic cell sorting (MACS) and anti-CD14 antibodies and differentiated over 12 days in culture in RPMI-1640 (plus 10% foetal bovine serum – FBS) into macrophages using 2 ng/ml GM-CSF, a protocol shown to produce macrophages with phenotypic and functional characteristics similar to lung macrophages[18, 19]. The GM-CSF containing media were then removed and replaced with media containing 10 ng/ml rhIL-4 or rhIL-13, and culture continued for a further 24 h. In order to check whether the differentiated cells displayed a M2 phenotype, culture supernatants were analysed by ELISA for CCL17 release whilst the resuspended cells were analysed for a panel of M2 biomarkers (CD206, CD163, CLEC10A/CD301, *Ccl17* and *Arg*1) using a combination of flow cytometry and RT-PCR (see online supplement).

Sampling of lung macrophages

Bronchoalveolar lavage (BAL) and sputum induction were performed as described[20]. Cells were isolated and immediately processed in 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 characterisation of lung macrophages was performed by flow cytometry using a 9-colour FACSAria cell sorter and FACSDiva Software (v5.0.3). 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, anti-human CD3 PE-Cy7 and anti-human HLA-DR APC-Cy7. Using this protocol, CD45+CD3-HLA-DR+ cells were >90% pure macrophages, as shown by cytological analysis of sorted cells (Figure E1 in online supplement).

Separate aliquots were additionally incubated in a cocktail of antibodies specific for surface biomarkers (anti-CD206 APC and anti-CD14 PerCP-Cy5.5 or anti-CD301 AF647 and anti-CD163 PerCP-Cy5.5) 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. These were further washed in 1 × Perm/Wash and incubated with anti-human CD68-PE, anti-human TNF APC, anti-IL-10 AF647 or isotype control.

RT-PCR

RNA was extracted from FACS sorted sputum and BAL macrophages using a Stratagene Microprep Kit. Reverse transcription was carried out using a nanoScript Reverse Transcriptase kit. PCR amplifications for M2 biomarkers (*Ccl17, Clec10A, Arg1)* were performed on a BioRad iCycler using Precision 2× qPCR Mastermix and PerfectProbe® primers (for full sequences see online supplement). Gene expression was normalized to $_2$-microglobulin gene expression and quantified using the C_T method[21].

Analysis of cytokine release by MDM and BAL cells

MDM from 5 subjects were cultured in RPMI-1640 (+10% FBS) for 24 h and stimulated with 10 ng/ml IL-4 in the presence or absence of a range of concentrations of fluticasone propionate or PI3Kinase inhibitors. Similarly, BAL cells (from 13 asthmatics), composed of a median of 75% macrophages (for full differential cell counts see online supplement), were cultured in AIM V medium 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 h, supernatants were retained for cytokine and chemokine analysis.

Culture supernatants were analysed by a combination of ELISA and Luminex assay for IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF , IFN , CCL17 and CCL22 (for full details see online supplement).

Statistics

Statistical analyses were performed using a Kruskal-Wallis or Friedman ANOVA test with a Dunn's Multiple Comparison test or a Mann-Whitney U test or Wilcoxon's signed rank test as appropriate (GraphPad Prism v3, GraphPad Software Inc., San Diego, USA). Results were considered significant if p<0.05.

Results

Assessment of M2 biomarkers using monocyte-derived macrophages (MDM)

Both IL-4 and IL-13 significantly increased *Ccl17* and *Clec10a* gene expression (Figure 1A), with a trend towards increased *Arg1* gene expression (data not shown) induced by IL-13. Th2 cytokine-treated MDM (i.e. M2 MDM) also expressed significantly more HLA-DR and CD206 (Figure 1B) and less CD14 and CD163 on their surface (Figure 1B), with no difference in CLEC10A (CD301) or CD36 expression (data not shown). Small amounts of CCL17 were released from unstimulated MDM (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 macrophages

The M2 cell surface biomarkers identified above were applied to sputum and BAL macrophages from asthmatic patients and controls and analysed by multichromatic flow cytometry. The only surface biomarker that was differentially expressed was CD163 (Figure 2 and Table 2). Similar to M2 MDM, its expression was reduced in asthma when compared to health on macrophages in sputum (Kruskal-Wallis p=0.0346) but not in BAL (Kruskal-Wallis p=0.2893). Intracellular TNF and IL-10 expression was also not different (Table 2), 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 using the above method of selection was considered insufficient for gene expression analysis as any small contamination with other cell types could be amplified by PCR. Therefore, we added to the macrophage detection protocol the Lineage 1 (Lin) antibody cocktail (BD Biosciences) to exclude dendritic cells from the analysis. Cells sorted in this way (CD45+CD3-Lin+HLA-DR+) (Figure 3) from both sputum and BAL were on average 98% pure macrophages as ascertained by histochemical and morphological analysis of cytospins. 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 controls expressed little or no *Ccl17* mRNA (median value 0), while significantly greater expression was observed in both mild (median value 3.421) and moderate asthmatics (median value 7.63). The *Ccl17* expression correlated weakly (r^2=0.1493) but significantly (p=0.0252) with sputum eosinophilia (Figure 4) but not with neutrophil counts ($r^2 = 0.04$). When these analyses were performed on individual asthma groups (MA r^2=0.279 p=0.063, MO r^2=0.016 p=0.7149) and healthy controls

(r^2=0.061 p=0.1456) separately, none of these correlations was significant, nor was there a significant correlation when asthmatics were analysed as a group in the absence of controls (r^2=0.109, p=0.1331), suggesting the correlation is probably "pulled" into significance because of the inclusion of low CCL17 expressing, low eosinophil healthy controls. Although a significant increase in *Ccl17* mRNA expression by BAL cells was observed (Kruskal-Wallis p=0.0449), in contrast to the sputum data, this increase was only significant when comparing mild and moderate asthmatics by Dunn's post-hoc test. There was a trend towards increased expression of *Clec10a* mRNA in both sputum and BAL macrophages from mild but not moderate asthmatics, although this was not statistically significant (data not shown).

Cytokine production by BAL cells

To ascertain whether increased expression of *Ccl17* mRNA in macrophages translated into increased release of CCL17 protein, freshly isolated BAL cells (containing a median of 75% macrophages) were cultured for 24 h and supernatants analysed for CCL17 by Luminex assay. The lack of difference in CCL17 *ex vivo* release by BAL cells observed (online supplement Figure E4) suggested no correlation between the *in vivo* gene expression and the *ex vivo* expression of CCL17. Additional cytokine measurements were also performed in order to assess whether macrophages released a predominance of Th1 or Th2 cytokines. None of the Th2 (IL-4, IL-5 and IL-13) or Th1 (IFN- , IL-2 and IL-12) cytokines were detected, while the previously reported M1 (TNF, IL-6, IL-8) and M2 (IL-10 and CCL22) biomarkers[5] were released at similar levels in all groups (online supplement Figure E4).

Inhibition of CCL17 release by the PI3Kinase inhibitor, LY294002, but not corticosteroids

The increased *Ccl17* mRNA expression observed in lung macrophages from moderate asthmatics on inhaled steroids suggested that the production of this chemokine may 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 were conducted with M2 MDM where fluticasone propionate had no effect on the CCL17 release in response to 10 ng/ml IL-4 (Figure 5A). We then studied whether inhibition of PI3Kinase, the enzyme suggested to be required for differentiation of monocytes into M2 macrophages[23], could reduce CCL17 release. The pan-PI3Kinase inhibitor, LY294002, caused a concentration-dependent inhibition (EC_{50} = 4.2 μM) that was significant at 10 μM (Figure 5A) with no significant LDH release (data not shown). Similar results were obtained with 10 ng/ml IL-13 (data not shown). In contrast, the specific PI3Kinase- inhibitor, IC81174, had little effect on the amount of CCL17 released by IL-4 stimulated-MDM except at the highest concentration used (Figure 5A) that caused an increase in LDH release (data not shown). Similarly, the spontaneous release of CCL17 by BAL cells from asthmatics (n=13) was significantly inhibited by 10 μM LY294002 from mean 14.82 pg/ml to 7.49 pg/ml (Figure 5B).

Discussion

In contrast to animal models of asthma, this study has demonstrated only a partial M2 macrophage phenotype in asthmatic airways, consisting of increased *Ccl17* mRNA expression and reduced CD163 cell surface expression when compared to health. Whilst blood monocytes did not express detectable amounts of *Ccl17* mRNA (data not shown), raised 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 Th2 type inflammation[24], providing a mechanism for increased recruitment of CCR4+ cells (eosinophils and Th2 lymphocytes) for which CCL17 is a ligand. The finding of persistently raised *Ccl17* mRNA in macrophages

from both sputum and BAL of moderate asthmatics on inhaled corticosteroids and the inability of corticosteroid to inhibit *in vitro* CCL17 release by M2 MDM suggests that CCL17 production is relatively corticosteroid insensitive. Finally, our study suggests that the production of CCL17 is regulated by PI3Kinase.

Studies of macrophages in asthma to date 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 asthma 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 asthma. Another study of M1/M2 macrophages in 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 COPD smokers and a reciprocal decrease of M1 phenotype gene expression[33]. However, the profile of differentially expressed M2 markers (e.g. Adenosine A3 receptor, MMP2, MMP7) was different from our study, while CCL17 and CD163 were not differentially expressed in either healthy smokers or COPD smokers.

Interpretation of the literature is complicated by differences in the definition of M2 phenotypes based on the use of different M2-polarising protocols, applying either IL-4/ IL-13[5], IL-10[7] or M-CSF[34], all of which produce slightly different macrophage phenotypes. Atopic asthma is considered to involve Th2 cytokines[2], so in keeping with the M2 phenotype described by Martinez et al (2009)[5], we have used IL-4 and IL-13 stimulated MDM as our M2 benchmark.

In the current study, CCL17 and CD163 expression varied considerably between the subjects studied, 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, which sample 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 following 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 Th2 T cell recruitment[12]. Stimulation of bronchial tissue explants by allergen induced CCL17 release in steroid-naive asthmatics but, of more relevance to the current study, spontaneous release was raised in explants from moderate asthmatics on inhaled corticosteroids. Furthermore, the numbers of sputum and blood CCR4+ T cells were significantly raised in asthmatics on high-dose inhaled and oral corticosteroids. Taken together with the current findings that *Ccl17* mRNA expression is persistently high in corticosteroid-treated asthmatics and that *in vitro* IL-4-induced CCL17 release by M2 MDM cannot be suppressed by corticosteroids, this study adds to accumulating evidence that CCL17 is an important chemokine in asthma whose production is relatively corticosteroid insensitive.

Increased expression of CD163, a haptoglobin-haemoglobin scavenger receptor[37], has been associated with the M2 phenotype *in vitro*[5, 7, 10, 38]. While most studies showed a role for IL-4 and IL-13 in driving this phenotype, Tiemessen et al. showed it to depend on IL-10 and not IL-4/IL-13[7]. In contrast to the published *in vitro* studies, we have found that CD163 expression is decreased in asthma. The finding that MDM exposed to IL-4 and IL-13

reduced CD163 expression suggests that Th2 cytokines may be responsible in asthma. In agreement with our study, Van den Heuvel et al. (1999) demonstrated that IL-4 treatment decreases CD163 expression, although when added to cells pre-treated with glucocorticoids, it increases the expression[22]. In contrast, in our study the reduced expression of CD163 on asthmatic sputum macrophages 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 PI3Kinase, 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 PI3Kinase. At a concentration of 10 μM this compound was effective at inhibiting CCL17 release from both cultured BAL cells and IL-4-stimulated MDM. Although this suggests the involvement of PI3Kinase, it is recognised that at concentrations. >10 μM LY294002 inhibits other kinases[40]. Previous studies have suggested that inhibiting the PI3Kinase- isoform is effective at reducing airway inflammation and hyperresponsiveness in murine models of asthma[41]. Furthermore, inhibition of PI3Kinase- using theophylline reverses the steroid insensitivity of COPD macrophages[42]. However, the specific PI3Kinase- inhibitor, IC87114, had little effect on IL4-induced CCL17 release from MDM, suggesting that, in contrast to the murine model[23], an isoform other than PI3Kinase- is involved in IL-4-induced CCL17 production in human macrophages. In summary, this study suggests involvement of the PI3Kinase 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 MDM and there have been reports of increases on macrophages in 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 towards increased CLEC10a mRNA but not CD301 (the protein marker of CLEC10a) in macrophages in asthma. Future studies will need to look for internalized or intra-cellular 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 partly, account for the lack of difference in CCL17 protein release by BAL cells *ex vivo*. Other cell types, although they constituted on average 30% of total BAL cells, may have bound some of the cytokines/chemokines released in culture (e.g CCL17 by CCR4+ cells). Furthermore, contact with plastic may have altered the BAL macrophage phenotype[46]. Similarly, the *in vivo* conditions required for the phenotype to be detected may 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 individuals. When studying macrophage function, stimuli are often used, but we chose not to expose the BAL cells to any exogenous stimuli such as LPS because this could skew responses towards a 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 asthma remains unclear. It is possible that IL-4 and IL-13 do still 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 Stat6 may not be involved in the development of the CCL17-producing macrophages. The promoter for CCL17 also contains NF- B motifs[47, 48] and the archetypal NF- B activator, TNF, induces expression of *Ccl17* mRNA in human bronchial epithelial

cells[49]. Infection of murine lung epithelium with respiratory syncytial virus (RSV) 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 via an EGFR/NF-κB-dependent pathway[16]. As both RSV and Der p 1 are implicated in asthma[52, 53], these agents may activate macrophages via NF-κB-dependent pathways to produce CCL17 in a steroid-independent manner. This study has involved a mix of eosinophilic and non-eosinophilic 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

ACQ	Asthma control questionnaire
AF647	Alexa Fluor 647
ANOVA	Analysis of variance
APC	Allophycocyanin
APC-Cy7	Allophycocyanin-Cyanine 7
Arg1	Arginase 1
BAL	Bronchoalveolar lavage
CCL	CC Chemokine Ligand
CCR	CC Chemokine Receptor
CD	Cluster of differentiation
CLEC10A	Macrophage galactose C-type lectin (CD301)
COPD	Chronic obstructive pulmonary disease
Der p	Dermatophagoides pteronyssinus
EGFR	Epithelial Growth Factor receptor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Foetal Bovine Serum

FEV1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
HC	Healthy control
HLA-DR	Human leukocyte antigen DR
Lin 1	Lineage 1 antibody cocktail
LPS	Lipopolysaccharide
M1	Classically-activated macrophages
M2	Alternatively-activated macrophages
MA	Mild asthmatic
MACS	Magnetic-assisted cell sorting
M-CSF	Macrophage Colony Stimulating Factor
MDM	Monocyte-derived macrophage
MO	Moderate asthmatic
mRNA	messenger Ribonucleic Acid
NF-κB	Nuclear factor κB
PC$_{20}$	Provocative concentration that induces a 20% drop in FEV$_1$
PE	Phycoerythrin
PE-AF610	Phycoerythrin-Alexa Fluor 610
PE-Cy7	Phycoerythrin-Cyanine 7
PerCP-Cy5.5	Peridinin Chlorophyll Protein-Cyanine 5.5
PI3Kinase	phosphatidylinositol-3-kinase
rh	recombinant human
RSV	Respiratory Syncytial Virus
RT-PCR	Reverse transcription-polymerase chain reaction
Stat	Signal transducer and activator of transcription
Th	T helper
TNF	Tumor Necrosis Factor

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Key message

- Lung macrophages in asthma do not express biomarkers associated with the alternative, M2 phenotype reported in studies of monocyte-derived macrophages and animal models.

- Lung macrophages of asthmatic patients express more CCL17 and less CD163 and the former is associated, albeit weakly, with sputum eosinophilia and cannot be suppressed by corticosteroids but can be attenuated by PI3Kinase inhibition.

- Macrophage expression of CCL17 may contribute to the airway inflammation observed in asthma through recruitment of cells expressing CCR4, the cell surface receptor for CCL17.
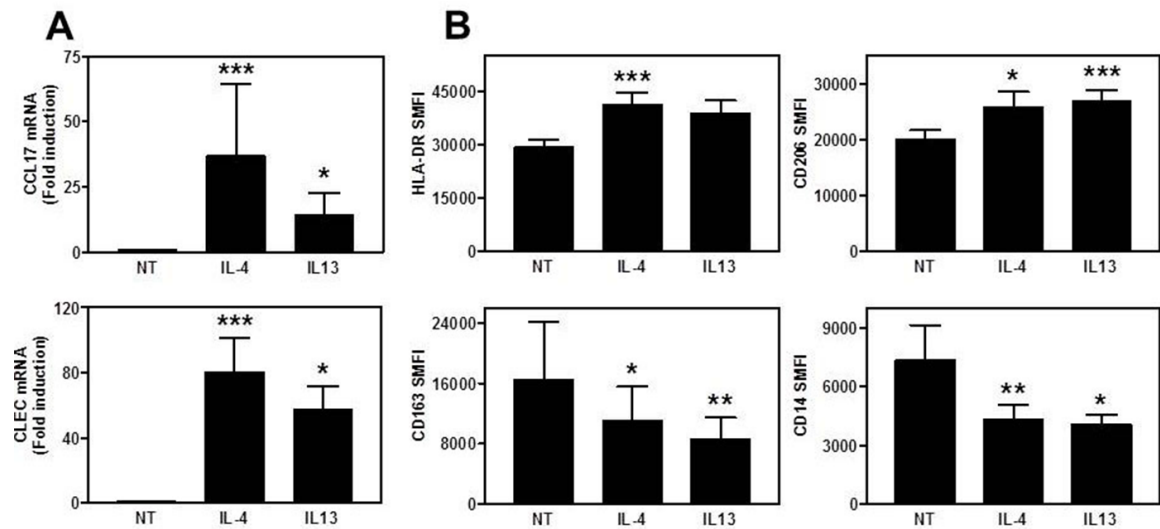
FIGURE 1. Expression of biomarkers on MDMs stimulated with IL-4 or IL-13.
A) Gene expression of *Ccl17* and *Clec10A* are expressed as fold induction compared to NT
(mean±SE, n=11). **B**) Cell surface expression data are expressed as specific mean
fluorescent intensity (SMFI - mean±SE, n=12). Data analysed using Friedman ANOVA and
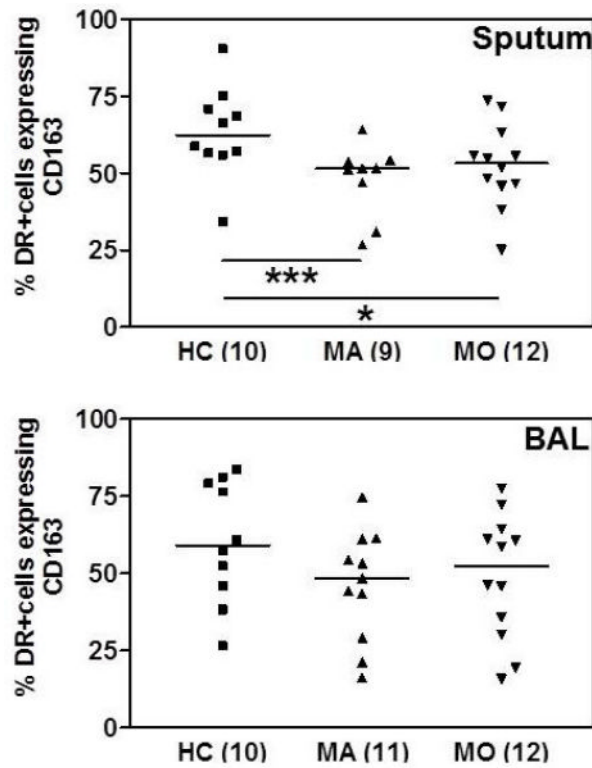Dunn's multiple comparison test compared to NT. * p<0.05, ** p<0.01, *** p<0.001

FIGURE 2. Analysis of lung macrophage M2 marker expression by flow cytometry.
Expression of cell surface CD163 on sputum and BAL macrophages of healthy controls
(HC), mild asthmatics (MA) or moderate asthmatics (MO). Bars indicate median values,
numerals in parentheses indicate *n*. Data are expressed as percentage of Live+CD45+CD3-
HLA-DR+ cells and analysed by Kruskal-Wallis ANOVA and Dunn's Multiple Comparison
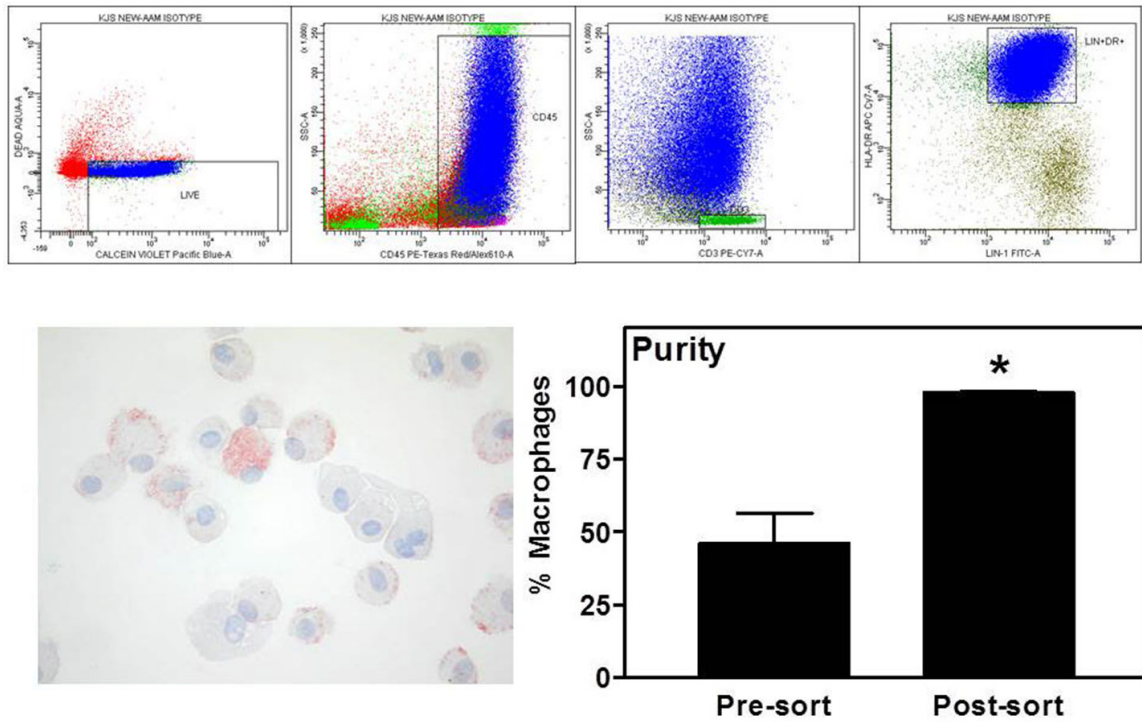test. * $p < 0.05$, *** $p < 0.001$.

FIGURE 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+Lin1+ events were sorted into PBS using a FACSAria and analysed by cytospin
(representative micrograph shown × 40 original magnification). Data are expressed as a
percentage of total cells, presented as mean±SE (n=6) and analysed using a one-tailed
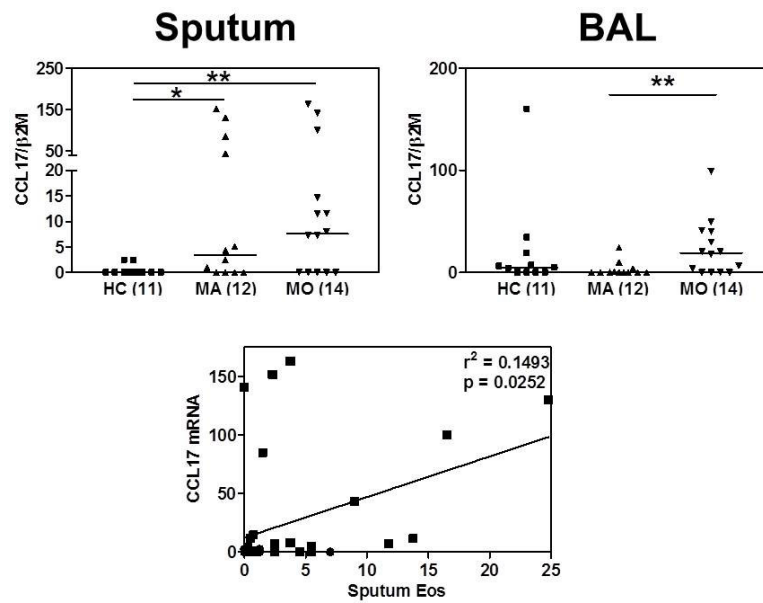Wilcoxon signed rank test. ** p<0.01

**FIGURE 4. CCL17 mRNA levels (normalised to 2-microglobulin) and association with sputum eosinophil counts.**

Bars indicate median values and numerals in parentheses indicate *n*. Data were analysed using Kruskal-Wallis ANOVA followed by Dunn's Multiple Comparison test. * p<0.05, ** p<0.01 The association between CCL17 mRNA expression and percentage sputum eosinophils was analysed by Spearman rank correlation test (HC Asthma).
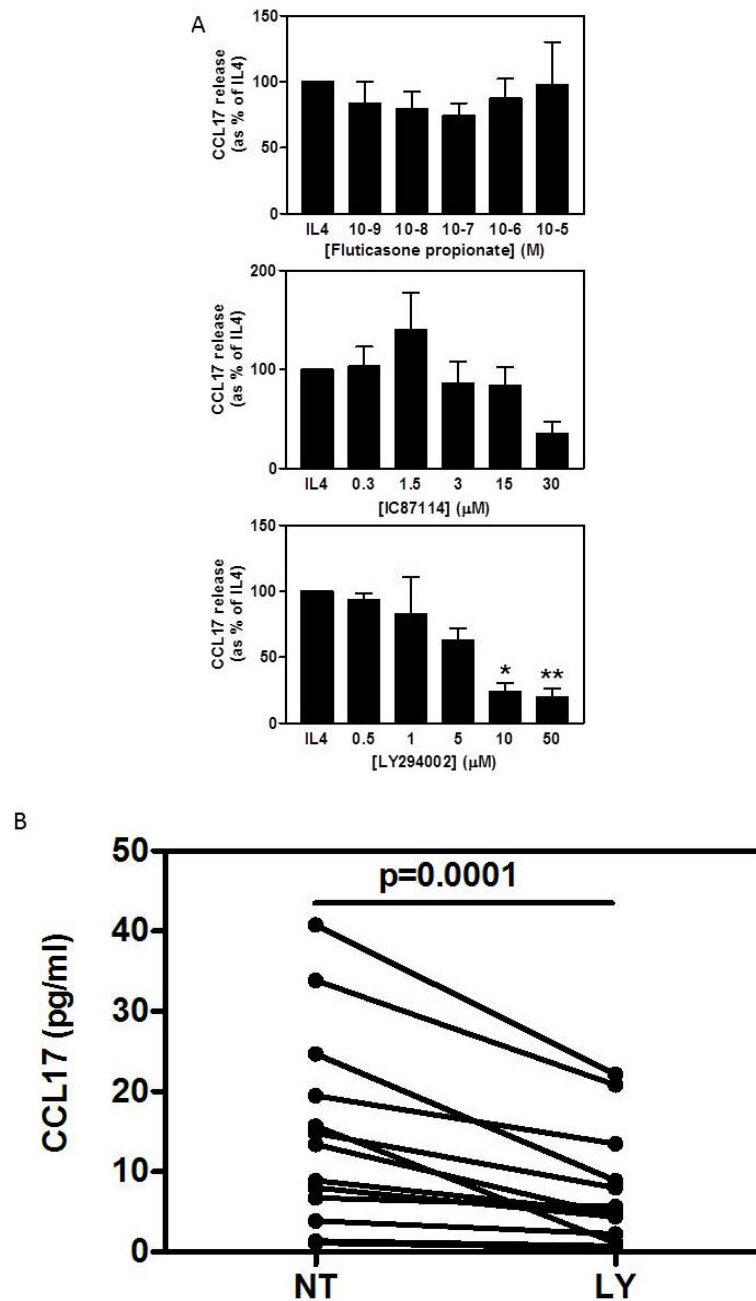
FIGURE 5.
A) Effects of fluticasone propionate and PI3Kinase inhibitors on CCL17 release by IL-4-stimulated MDM. Data expressed as percentage of the maximum IL-4 stimulation (mean ±SE, n=5). Data analysed by Friedman ANOVA and Dunn's multiple comparison test. **B**) Effect of LY294002 on spontaneous release of CCL17 from BAL cells (medians, n=13). Data analysed using one-tailed Wilcoxon signed rank test. * p<0.05, ** p<0.01, *** p<0.001

Table 1

Baseline characteristics of healthy control (HC), mild asthmatic (MA) and moderate asthmatic (MO) volunteers.

Data are expressed as median values (IQr) to 2 d. p. N.D. indicates not determined. Data were analysed using a Kruskal-Wallis test followed by a Dunn's Multiple Comparisons test. PC_{20} and ACQ data were analysed using a Mann-Whitney U test.

	HC	MA	MO
N	12	12	14
Sex (M/F)	5/7	6/6	6/8
Age	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	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	N.D.	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 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)

[**] p<0.01 versus HC,

[***] p<0.001 versus HC,

[†] p<0.05 versus MA,

[‡] p<0.01 versus MA,

[¶] p<0.001 versus MA.

Table 2

Cell surface and intracellular protein expression by macrophages from healthy controls (HC), mild asthmatics (MA) and moderate asthmatics (MO).

HLA-DR, CD14, CD301, TNF and IL10 are represented as specific mean fluorescence intensity (SMFI) and CD206 is shown as percentage of cells staining positively.. Data are expressed as median values (IQR).

	Sputum			BAL		
	HC	**MA**	**MO**	**HC**	**MA**	**MO**
Extracellular						
HLA-DR (SMFI)	59210 (44120 – 75520)	57050 (40780 – 76880)	46280 (42470 – 63470)	49140 (34100 – 56390)	46470 (36650 – 57300)	44470 (30760 – 61870)
CD14 (SMFI)	1920 (1498 – 3244)	1729 (1520 – 1993)	1932 (1489 – 2694)	1260 (662.5 – 1612)	1328 (717.5 – 1808)	1049 (660 – 1922)
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)	2785 (1439 – 5921)	2993 (1674 – 3852)	3208 (2008 – 3909)	2727 (1397 – 5295)	3314 (2917 – 4640)	2540 (1198 – 5318)
Intracellular						
>TNF (SMFI)	3781 (3174 – 4000)	4464 (3383 – 5366)	3867 (3379 – 4831)	6675 (5232 – 8380)	6717 (5172 – 8141)	6120 (5473 – 7148)
IL-10 (SMFI)	1296 (1005 – 1735)	1242 (1001 – 1900)	1615 (814.5 – 2472)	1324 (722 – 2229)	1976 (1700 – 2179)	1300 (920 – 2014)

Table 3

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	n.t.	n.t.
Ym1		No human homolog	n.t.	n.t.
Cell surface expression				
CD23			n.t.	n.t.
CD163	38	22		
CD206				No change
MHCII/HLA-DR				No change
Chemokine expression				
CCL13[*]			n.t.	n.t.
CCL14	No murine homolog		n.t.	n.t.
CCL17				
CCL18	No murine homolog		n.t.	n.t.
CCL22			£	No change
CCL24			n.t.	n.t.

[+]Information from Martinez et al.5 except where referenced differently.

[@]Data all from the current study. n.t. = not tested

£Data from the pilot of this manuscript obtained using a slightly different culture model (see online supplement).

[#]Murine equivalent is MGL1.

[*]Murine homolog of CCL13 is CCL2.