Transcriptional changes in alveolar macrophages from adults with asthma after allergen challenge

To the Editor,

Under homeostatic conditions, macrophages are the most abundant immune cells in the lung. Pulmonary macrophages are a heterogeneous cell population that can be classified in at least two distinct subpopulations, that is, interstitial macrophages, located within the lung parenchyma, and alveolar macrophages (AM) which reside in the airway lumen, allowing direct contact with the environment (eg, allergens, particulate matter, and commensal bacteria). In recent years, AM have been shown to play an important role in environmental allergen-induced airway inflammation in asthma. Elimination of resident AM resulted in enhanced type 2 airway inflammation in a mouse asthma model, while depletion of blood monocytes resulted in abrogation of newly formed AM after allergen challenge and a decreased type 2 immune response. Knowledge of phenotypic alterations of AM in allergic asthma in humans is limited. In this study, we investigated the effect of house dust mite (HDM) and lipopolysaccharide (LPS) challenge on the transcriptome of AM from patients with mild asthma. We have shown previously that intrabronchial HDM/LPS challenge induces a mixed eosinophilic and neutrophil airways inflammation in asthma patients. Therefore, we hypothesize that exposure of AM to HDM/LPS would upregulate genes associated with eosinophil and neutrophil signaling.

AM were harvested from asthma patients who participated in a double-blind randomized placebo-controlled trial investigating the effect of C1 inhibitor on allergen-induced airway inflammation. The Medical Ethics Committee of the Academic Medical Center, Amsterdam (the Netherlands), approved the study, and all patients gave their written informed consent. For the current analysis twelve patients (treated with placebo) with intermittent-to-mild asthma, defined according to the criteria of the Global Initiative for Asthma guideline, and HDM allergy, confirmed by a positive skin prick and radioallergosorbent test, were challenged via bronchoscopy with saline (as internal control) in one lung segment and HDM extract (50 biological units, Dermatophagoides pteronyssinus origin; Allergopharma, Zeist, the Netherlands), combined with LPS (75 ng; from Escherichia coli, Clinical Center Reference Endotoxin, kindly provided by Anthony Suffredini, National Institute of Health, Bethesda, MD, USA) in the contralateral lung segment. The addition of LPS to HDM mimics a natural allergen exposure as LPS is a widespread pollutant and coexisting with HDM in house dust. After seven hours, a bilateral bronchoalveolar lavage (BAL) was performed. BAL fluid was pooled (per lung subsegment) and centrifuged at 4°C and 400 g for 10 min. Cells were stained with CD45 phycoerythrin (PE)-CF594 and CD71 Brilliant Violet (BV)421 (both BD Biosciences). AM, defined as cells expressing CD45 and CD71, were analyzed.
were isolated by flow cytometry sorting using FACS Aria III (BD Biosciences) with high purity (>95%; gating strategy see Figure S1). This sorting strategy is a limitation of our study as it selects CD71 positive AMS and does not discriminate between different AM subsets. In addition, flow cytometry did not include other markers, precluding discrimination of macrophages subsets. Sorted AM were stored in RNA later (Qiagen) at −80°C until analysis. Total RNA was isolated using RNAeasy isolation kit (Qiagen) and quantified by Qubit® 2.0 (Life Technologies). Paired samples (AM from saline and HDM/LPS challenged lung segments of one individual) from eight patients had a RNA integrity number >6 and were selected for further analysis. RNA-seq libraries were prepared using the KAPA RNA
FIGURE 1  Genome-wide analysis of alveolar macrophage response to HDM/LPS challenge. (A) Principal component analysis (PCA) of the 24,555 transcripts showing explainable variances of PC1 20% and PC2 13% between saline or HDM/LPS samples (B) Volcano plot depicting the differences in gene expression following either saline or HDM/LPS exposure. Horizontal line represents the multiple-test adjusted significance threshold adjusted $p$-value ≤0.05; fold expression ≥1.2 and adjusted $p$-value ≤0.05; fold expression ≤−1.2 (C) Ingenuity pathway analysis of the canonical pathway gene expression profiles in HDM/LPS samples relative to saline samples. Red bars, overexpressed pathways; blue bars, under expressed pathways (D) Heatmap plot of the top significant genes between saline and HDM/LPS samples.
HyperPrep with Ribosome Erosion (Roche) according to manufacturer’s instructions and sequenced using the Illumina HiSeq4000 (Illumina) instrument. Sequences were evaluated for quality (FastQC version 0.11.5; Babraham Institute) and trimmed (Trimomatic version 0.36). The remaining reads were used to align against the Genome Reference Consortium human genome build 38 (GRCh38) using HISAT2 (version 2.1.0) with parameters as default. The HTSeq and DESeq2 methods are standard bioinformatics methods used to calculate and compare sequence counts, respectively. Pathway analysis was done using Ingenuity Pathway Analysis (QIAGEN bioinformatics) software. To control the false discovery rate, Benjamini and Hochberg adjusted p-values <0.05 demarcated significance. Sequence libraries are publicly available through the National Center for Biotechnology Information under the following accession number GSE144576. The baseline characteristics of these patients and the leukocytes differentiation in BAL fluid are depicted in Table 1. None of the patients had any symptom at the study day.

Principal component analysis of 24,555 transcripts (sequence counts ≥10) revealed clear separation of saline or HDM/LPS challenges (Figure 1A), showing that HDM/LPS induced a marked transcriptomic response in AM. Comparing HDM/LPS challenge to saline identified 1013 significantly altered transcripts (fold expression ≤−1.2 or ≥1.2), of which 485 were upregulated and 528 downregulated as consequence of HDM/LPS challenge (Figure 1B). Pathway analysis of upregulated genes revealed predominantly pro-inflammatory responses such as inducible nitric oxide synthase, Toll-like receptor, p38 mitogen-activated protein kinase, and interleukin (IL)-1, IL-8, and IL-17 signaling (Figure 1C). IL-1 signaling has been implicated in neutrophilic subtype of asthma, especially an increased IL-1β. Likewise, IL-17 has been identified as an important cytokine in the pathogenesis of asthma due to its capacity to promote neutrophilic airway inflammation, which is associated with a severe asthma phenotype. In agreement, our data show upregulation of CXCL1, a chemoattractant for neutrophils, suggesting that AM aid in neutrophil recruitment following HDM/LPS challenge (Figure 1D). In line with these findings, HDM/LPS challenge induced a marked neutrophil influx in the airways. Interestingly, genes associated with the anti-inflammatory IL-10 signaling pathway were also upregulated. This could be explained by heterogeneity among AM including both pro- and anti-inflammatory subpopulations and/or concurrent induction of both pro- and anti-inflammatory pathways in the same AM. This observation suggests a regulatory role for AM to maintain homeostasis by counteracting the pro-inflammatory processes inflicted by allergen challenge. Among downregulated transcripts were genes associated with cell cycle and growth (T-cell receptor, role of NFAT in cardiac hypertrophy), DNA damage and repair PI3/Pi4-kinase family, including ATM, PIK3CB and PIK3CG (Table S1) (breast cancer regulation by stathmin 1) and metabolic pathways (eg, insulin receptor and leptin signaling, Figure 1C,D). Network graphs of the most enriched genes are displayed in Figures S2 and S3.

To the best of our knowledge, this is the first investigation to examine gene expression profiles in AM from asthma patients challenged with HDM and LPS in vivo. Using a well-controlled study design allowing paired analysis of saline and HDM/LPS effects in the same patient, we identified different transcriptional profiles in AM as consequence of local exposure to HDM and LPS. These findings may provide a better understanding of AM functions in (exacerbations of) allergic asthma.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest to disclose.

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Mass cytometry reveals unique subsets of T cells and lymphoid cells in nasal polyps from patients with chronic rhinosinusitis (CRS)

To the Editor,

Chronic rhinosinusitis (CRS), a heterogeneous disease characterized by chronic inflammation in the nasal cavity and sinuses, causes significant morbidity and diminished quality of life while costing the U.S. health system $22–32 billion annually.\(^1\,2\) Aberrant activation of the immune system in nasal and sinus mucosa plays a key role in the etiology and pathophysiology of CRS. Indeed, CRS is associated with increased expression of type 2 and other cytokines in the tissues, and patients have been treated successfully by targeting these molecules.\(^3\) To better understand the pathophysiologic mechanisms of CRS, characterization of the immune cells that infiltrate nasal and sinus tissues is critical. While conventional tools, such as flow cytometry, have been useful for identifying specific immune cell types, the cellular complexity and functional diversity of the human immune system necessitate the use of high-dimensional tools to characterize heterogeneity of immune cells and to elucidate their roles in disease.\(^4\) Mass cytometry, similar to flow cytometry, is suited to performing single-cell analysis. Spectral overlap between channels limits the number of parameters that can be investigated simultaneously by flow cytometry. Mass cytometry uses monoisotopic heavy metals to tag antibodies and detection by inductively coupled plasma mass spectrometry (ICP-MS).\(^4\) The availability of many different isotopes increases the multiplexing capacity of mass cytometry to, theoretically, 135 separate channels. While mass cytometry supports traditional manual gating, the volume and multidimensionality of the data acquired necessitate development of algorithmic analyses that cluster cells by common expression profiles and enable discovery of previously unknown cell populations. This is likely to become a great advantage when analyzing tissue-resident cells, as unexpected cell population may be generated in response to the tissue environment. Thus, while mass cytometry is slower than flow cytometry and is unsuitable for cell sorting, it is comparable to flow cytometry in characterizing known cell types and is more robust for discovering new cell types. Here, we performed a proof of concept pilot study applying the mass cytometry technology to CRS research and characterized the T-cell and lymphoid cell populations infiltrating into nasal polyps.

Nasal polyps from 18 subjects with CRS with nasal polyps (CRSwNP) and control sphenoid mucosal tissues from four subjects without CRS were obtained (see Table S1 for subject demographics). Peripheral blood mononuclear cells (PBMCs) from healthy individuals without sinus disease provided another layer of controls. Tissue cells and PBMCs were stained with selected fluorescent-labeled antibodies or with the mass cytometry metal-labeled T-cell panel (Table S2). We first compared the performance of both methods using conventional biaxial plots (Figure S1). The ratio of CD4\(^+\) to CD8\(^+\) cells (Figure 1A) and proportions of CD4\(^+\), CD8\(^+\), double-negative (DN), and double-positive (DP) cells within the CD3\(^+\) T-cell compartment (Figure 1B) were similar within each cellular source whether analyzed by flow cytometry or by mass cytometry. Indeed, CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) frequencies were not statistically different based on cytometry methods used (p > 0.05 for both CD4\(^+\) and CD8\(^+\) cells in PBMC, control tissue and nasal polyp specimens).

Tissue-resident memory T (T_{EMR}) cells, which highly express the activation marker and adhesion molecule CD69, likely play a pivotal role in pathophysiology of mucosal organs in immune-mediated diseases.\(^5\) Conventional flow cytometry revealed a significantly greater proportion of CD4\(^+\) cells co-expressing CD69 in nasal polyps compared to control sinus tissues (Figure 1C, p < 0.05); few CD4\(^+\) T cells in PBMCs expressed CD69. Mass cytometry analysis showed comparable

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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