Identification of a distinct glucocorticosteroid-insensitive pulmonary macrophage phenotype in patients with chronic obstructive pulmonary disease

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Background: In patients with chronic obstructive pulmonary disease (COPD), pulmonary macrophages increase in number, release increased levels of inflammatory mediators, and respond poorly to glucocorticosteroids. Whether this is due to a change in macrophage phenotype or localized activation is unknown.
Objective: We sought to investigate whether macrophages from patients with COPD are a distinct phenotype.
Methods: Macrophage populations were isolated from human lung tissue from nonsmokers, smokers, and patients with COPD by using Percoll density gradients. Five macrophage populations were isolated on the basis of density (1.011-1.023, 1.023-1.036, 1.036-1.048, 1.048-1.061, and 1.061-1.073 g/mL), and cell-surface expression of CD14, CD16, CD163, CD40, and CD206 was assessed by using flow cytometry. Release of active matrix metalloproteinase 9, TNF-α, CXCL8, and IL-10 was measured by using ELISA.
Results: The 2 least dense fractions were more than 90% apoptotic/necrotic, with the remaining fractions greater than 70% viable. Macrophages from nonsmokers and smokers were $CD163^+$, $CD206^+$, $CD14^+$, and $CD40^-$, whereas macrophages from patients with COPD were less defined, showing significantly lower expression of all receptors. There were no differences in receptor expression associated with density. Macrophages from patients with COPD of a density of 1.036 to 1.048 g/mL released higher levels of active matrix metalloproteinase 9 compared with cells from nonsmokers, with no difference between the remaining fractions. This population of macrophages from patients with COPD was less responsive to budesonide compared with those from nonsmokers and smokers when stimulated with LPS. Glucocorticosteroid insensitivity was selective for proinflammatory cytokines because budesonide inhibition of LPS-stimulated IL-10 release was similar for all macrophages.
Conclusions: This study identifies a specific macrophage phenotype in the lungs of patients with COPD who are glucocorticosteroid insensitive with a density of 1.036 to 1.048 g/mL but do not correspond to the current concept of macrophage phenotypes. (J Allergy Clin Immunol 2014;133:207-16.)

Key words: *Matrix metalloproteinase 9, flow cytometry, CD163, CD206*

Inflammation is a prominent feature of chronic obstructive pulmonary disease (COPD), which is primarily caused by cigarette smoking in industrialized nations but driven by indoor pollution in the developing world.[1] Macrophages might play a pivotal role in driving this underlying inflammation,[2] which subsequently leads to tissue damage, fibrosis of the small airways, chronic bronchitis, and emphysema.[3,4] This inflammation is insensitive to glucocorticosteroid therapy,[5] which might, in part, be due to macrophages.[6,7]

Macrophages can be classically activated after priming with IFN-γ and stimulation with LPS or alternatively activated by exposure to IL-4 or IL-13.[8,9] These distinct macrophage activation or polarization states were first described almost 4 decades ago[10,11] and might reflect different macrophage phenotypes.[12] These phenotypes have been defined, with M1 corresponding broadly to classically activated and M2 corresponding to alternatively activated.[13] However, differences in macrophage responses have been further observed within the M2 phenotype, resulting in the subclassifications of M2a, M2b, and M2c, which are induced by IL-4/IL-13, immune complexes/LPS, and IL-10, respectively.[13] However, these phenotypes might not be fixed and might switch depending on the local microenvironment.

It is possible that the highly activated pulmonary macrophages in patients with COPD might represent a shift in phenotype. Alveolar macrophages from cigarette smokers show increased expression of M2-like related genes, whereas cells from patients with COPD showed a suppression of classically activated M1-like related genes.[14] In contrast, others have shown that active smoking in patients with COPD reduced macrophage phagocytic ability and expression of CD163 and inferred a predominance of proinflammatory macrophages.[15] In the lung the presence of different macrophage populations has been well known, and subpopulations of macrophages have been identified on the basis of density by using discontinuous Percoll gradients.[16-19] It has been suggested that these differences in density are related to

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Abbreviations used
APC: Allophycocyanin
COPD: Chronic obstructive pulmonary disease
EC_{50}: Median effective concentration
FITC: Fluorescein isothiocyanate
MFI: Median fluorescence intensity
MMP: Matrix metalloproteinase
PE: Phycoerythrin
PerCP: Peridinin-chlorophyll-protein

stages of cell differentiation, maturation, or activation states,[13,17] although whether they reflect different phenotypes is unknown. Increasingly, macrophage phenotypes are being investigated in murine models and identified by using specific cell-surface receptor expression.[20-23] However, in human subjects reliable cell-surface markers of the classically activated macrophage phenotype are not well defined; nevertheless, CD40 is predominantly used,[23,24] with the scavenger receptor CD163 and the mannose receptor CD206 used for alternatively activated M2 macrophages.[23,25,26] Therefore this study compared expression of phenotypic markers and release of inflammatory mediators from human lung tissue macrophages from nonsmokers, smokers, and patients with COPD isolated by using density gradients to identify a specific COPD population that might be responsible for the glucocorticosteroid insensitivity of the inflammatory response observed in this disease.

METHODS

Subject selection

Lung tissue surplus to diagnostic requirements was obtained from pulmonary resections at the Royal Brompton and Harefield NHS Foundation Trust. Smokers had a smoking history of at least 10 pack years, and patients with COPD were stable and fulfilled the American Thoracic Society criteria.[27] All subjects provided written informed consent, as approved by the London-Chelsea Research Ethics Committee. There were significant differences between FEV_1 in liters, FEV_1 percent predicted, and FEV_1/forced vital capacity ratio between smokers and patients with COPD compared with nonsmokers but matched for age and smoking history (Table I).

Isolation of lung tissue macrophages

Lung tissue macrophages were isolated, as described previously, by using discontinuous Percoll density gradients.[28] Cells were collected from the interface of 10% to 20% (vol/vol), 20% to 30% (vol/vol), 30% to 40% (vol/vol), 40% to 50% (vol/vol), and 50% to 60% (vol/vol) Percoll layers, corresponding to densities of 1.011 to 1.023, 1.023 to 1.036, 1.036 to 1.048, 1.048 to 1.06, and 1.061 to 1.073 g/mL. Macrophage purity was confirmed by using the Reastain Quick-Diff Kit (Genatur, London, United Kingdom), according to the manufacturer's instructions, and anti-CD68 staining, as previously described.[29] Additional detail is provided in the Methods section in this article's Online Repository at www.jacionline.org.

Measurement of viability

Lung tissue macrophages isolated from the interface of each Percoll gradient were stained with Annexin V–phycoerythrin (PE)/7-aminoactinomycin D by using the Annexin V apoptosis detection kit, according to the manufacturer's instructions (Becton Dickinson, Oxford, United Kingdom). The reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide to formazan was measured colorimetrically to assess the effect of experimental conditions on cell viability. Untreated cells were considered 100% viable. None of the treatments used in this study altered cell viability.

Measurement of cell-surface markers

After exclusion of dead cells, doublets, contaminating T cells, and dendritic cells, HLA-DR^+ cells were gated and separated according to the expression of CD14 and CD16, followed in sequence by the expression of CD163, CD40, and CD206. Quadrant gates were set by using the appropriate fluorescence minus one control. Additional details on this method and an example of the gating strategy are available in the Methods section and Fig E1 in this article's Online Repository at www.jacionline.org. Data are expressed as the percentage of cells that express the protein being investigated and as relative fluorescence intensity, which was calculated based on the fluorescence values (median channel) of the cells stained with antibody divided by the fluorescence values (median channel) for the respective control (median fluorescence intensity [MFI]).

Measurement of active matrix metalloproteinase 9

Release of active matrix metalloproteinase (MMP) 9 into cell culture media was measured by using a commercially available kit, according to the manufacturer's instructions (R&D Systems, Abingdon, United Kingdom).

Measurement of CXCL8, TNF-α, and IL-10 by using ELISA

Macrophages were seeded onto 96-well plates at a density of 10^5 cells per well and allowed to adhere overnight. Cells were pretreated for 1 hour with budesonide at the concentrations indicated, followed by stimulation with a submaximal concentration of LPS (10 ng/mL; *Salmonella enterica* serotype enteritidis) for 20 hours, as described previously.[30] Cell-free supernatants were removed, and CXCL8, TNF-α, and IL-10 levels were measured by using ELISA (R&D Systems). The lower limit of detection for these assays was 31 pg/mL.

Statistics

Comparisons were conducted by using Kruskal-Wallis tests, followed by Dunn postcorrectional tests or Wilcoxon signed-rank tests as appropriate, with a P value of less than .05 considered significant. Median effective concentration (EC_{50}) values were analyzed by using nonlinear regression analysis. All analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, Calif).

RESULTS

Approximately 90% of cells extracted from the 10% to 20% (vol/vol) and 20% to 30% (vol/vol) Percoll interfaces were nonviable, resulting in their exclusion from further analysis (Table II). Cell viability increased with increasing cell density, with greater than 70% of cells viable in the remaining cell fractions (Table II). Cells from the 3 remaining viable fractions had clearly defined nuclei and cytoplasm reminiscent of macrophages with no clear morphological differences (see Fig E2 in this article's Online Repository at www.jacionline.org) and were confirmed to be derived from a macrophage lineage by means of positive staining with anti-CD68 (see Fig E3 in this article's Online Repository at www.jacionline.org). The distribution of macrophages across the separate fractions was similar regardless of the origin of the tissue (see Table E1 in this article's Online Repository at www.jacionline.org), and the number of macrophages isolated per gram of tissue was also similar, although significantly more macrophages were isolated from the 50% to 60% (vol/vol) interface of tissue from smokers compared with nonsmokers and patients with COPD (see Table E1).